

REMARKS

The Office Action

Claims 1-7 were pending and under examination at the time of the Office Action. A certified translation of the foreign priority document is required. Claim 3 stands prospectively objected to under 37 C.F.R. § 1.75. Claims 1-7 stand rejected under 35 U.S.C. § 102(b). Applicants address each objection and rejection in turn below.

Claim Amendments

Claim 1 has been amended to recite “non-immortalized” T cells. Support for this amendment is found, for example, on page 12, lines 31-32 of the English language specification, which states (emphasis added): “...safety issues such as transformation and immortalization due to chromosomal abberation[sic] **do not occur.**” Claims 3-7 have been cancelled. New claims 8-21 have been added. Support for new claim 8 is found, for example, in the Examples (page 34, line 6 to page 38, line 3) and in Figures 1-4, 6, and 8-10 (unless noted otherwise, all references to the specification refer to the English language specification). Support for new claims 9 and 11 is found, for example, on page 2, lines 8-14, and on page 5, lines 17-22 of the specification (emphasis added):

Page 2, lines 8-14

To search for vectors that can efficiently transduce genes into T cells, vectors were transferred into T cells under various conditions and gene transfer efficiency was measured. As a result, the present inventors discovered that paramyxovirus vectors have a high gene transduction efficiency towards **antigen-activated T cells**. The gene transduction was specific to **antigen-activated T cells**, that is, the vectors' gene transduction efficiency towards

activated T cells was remarkably higher compared to naive T cells. Paramyxovirus vectors can be preferably used as vectors for gene transduction into **antigen-activated T cells**.

Page 5, lines 17-22

Further, **activated T cells in this invention are preferably T cells activated by antigens**. Gene transduction via Sendai virus vectors is selective for antigen-activated T cells. Transduction efficiency is low in the case of antigen-nonspecific T cells, which are bystander-activated from specific T cells that have responded to antigens *ex vivo*. Therefore, vector-mediated gene transduction efficiency can be dramatically improved by activating T cells with antigens, or by performing an equivalent activation.

Support for new claims 10 and 12 is found, for example, in the passages of the specification indicated below (emphasis added):

Page 26, lines 24-25

Fig. 3 is a series of dot plots representing the gene transduction efficiency under **alloantigen-specific activation** of naive T cells and T cell lines.

Page 33, lines 5-6

To assess the gene transduction efficiency under **alloantigen-specific activation** of naive T cells, fresh lymphocytes isolated from 2C-tg mouse were used.

Page 34, lines 17-19

Next, to examine whether it is possible to transduce genes into antigen-activated T cell lines, **an alloantigen was used** as a T cell-stimulating antigen, to which naive lymphocytes can respond and proliferate in primary cultures without in vivo immunization.

Page 36, lines 14-16

In the human **alloantigen-stimulated T cell line**, both CD4 and CD8 T cells showed exceptionally efficient gene transduction, 97% and 98% respectively, in the presence of immobilized antibodies (Fig. 8).

Support for new claim 13 is found, for example, on page 6, lines 15-19 and 30-33, and in Examples 1-3 (page 34, line 6 to page 36, line 17) of the specification. Support for new

claim 14 is found, for example, in original claim 5. Support for new claims 15 and 16 is found, for example, on page 2, lines 11-14; page 3, lines 6-11; and page 4, lines 23-33 of the specification. Support for new claim 17 is found, for example, in original claim 1; page 2, lines 12-13; and page 3, lines 6-8 and 20-21 of the specification (as shown below).

Page 2, lines 12-13

...gene transduction efficiency towards activated T cells was remarkably higher compared to naive T cells.

Page 3, lines 6-8

The present inventors discovered that this paramyxovirus vector is capable of transducing a gene into activated T cells with an extremely high efficiency.

Page 3, lines 20-21

The step of activating T cells enables efficient gene transduction by paramyxovirus vectors.

Support for new claims 18-21 is found, for example, as indicated above for new claims 8-10 and 13.

The present amendments were made solely to expedite prosecution, and applicants reserve the right to pursue any cancelled subject matter in this or a continuing application. No new matter has been added.

Priority

The Office states (page 2) that an English translation of the priority document has not been provided. Applicants submit herewith a verified translation of priority document

Japanese Patent Application No. 2002-310053 signed by the translator.

The Office has requested (page 2) that applicants indicate where support for each independent claim is found in the translation of the priority document. Support for claim 1 as presently amended is found, for example, on page 3, lines 30-31, and page 13, lines 34-35, of the translation of the priority document. Support for new claim 14 is found, for example, on page 4, lines 3-4, of the translation. Support for new claim 16 is found, for example, on page 3, lines 13-16; page 4, lines 12-17; and page 5, line 33 to page 6, line 7 of the translation. Support for new claim 17 is found, for example, on page 3, lines 14-15 and 30-31, and page 4, lines 12-14 and 27-28, of the translation.

In view of applicants' submission of a verified, signed translation of the priority document and indication of support for each independent claim in the priority document, applicants submit that the claims are entitled to benefit of the priority date.

Prospective Objection Under 37 C.F.R. § 1.75

The Office states that, should claim 1 be found allowable, claim 3 will be objected to under 37 C.F.R. § 1.75 as being a substantial duplicate thereof. In view of the cancellation of claim 3, the prospective objection under 37 C.F.R. § 1.75 is moot.

Rejection Under 35 U.S.C. § 102(b)

Claims 1-7 stand rejected under 35 U.S.C. § 102(b) as being anticipated by Yu et

al. (Genes to Cells, 2:457-466, 1997; “Yu”). The Office states (page 3):

Since Yu et al. teach a method of transducing a gene into activated T cells by contacting the activated T cells with a paramyxovirus vector carrying the gene, wherein the paramyxovirus vector is a Sendai virus vector, the claimed invention is anticipated by the above-cited art.

Applicants respectfully disagree. Yu describes Sendai virus-mediated gene transduction of two T cell lines, MT4 and Molt 4, stimulated with phytohemagglutinin (PHA) (see page 464, column 1, last paragraph, and Table 1 of Yu). MT4 is an acute lymphoblastic leukemia cell line transformed with human T-cell leukemia virus type 1, as shown in Exhibit 1 (data sheet from the National Institutes of Health AIDS Research & Reference Reagent Program). Similarly, Molt 4 is a leukemic cell line derived from a patient suffering from an acute lymphoblastic leukemia in relapse, as shown in Exhibit 2 (an additional National Institutes of Health data sheet). These cells are distinct from the T cells featured in the claims as presently amended. The present invention does not relate to the method of introducing a gene to leukemic cells, but rather a method of introducing a gene to non-immortalized T cells. Mouse T cells used in the examples of the specification have been obtained from spleen and lymph nodes (for example, see page 31, lines 13-17, of the specification). These T cell are not leukemic cells but non-immortalized T cells. The human T cells featured in the specification are also non-immortalized T cells obtained from peripheral blood of healthy donors (for example, see page 31, lines 17-19, of the specification). For purposes of clarification, claim 1 has been amended to recite “non-immortalized T cells” and thus is not anticipated by Yu.

In sum, Yu does not teach or suggest any method of transducing a gene into non-immortalized T cells. Furthermore, Yu does not teach or suggest activating non-immortalized T cells in order to elevate the efficiency of gene transduction. Thus, the presently claimed invention is neither taught nor suggested by Yu. The rejection under 35 U.S.C. § 102(b) should be withdrawn.

In addition, applicants submit that new claims 8-21, for the reasons stated above, are also free of the anticipation rejection over Yu.

To further illustrate the distinction between immortalized and non-immortalized cells, applicants direct the Office's attention to Exhibit 3 (Thenet et al., J. Cell. Physiol. 150:158-167, 1992; "Thenet") and Exhibit 4 (Jat et al., Proc. Natl. Acad. Sci. U.S.A. 88:5096-5100, 1991; "Jat"). Thenet describes the establishment of an immortalized rabbit articular chondrocyte cell line and states (abstract): "...the resulting cell lines displayed an apparently irreversibly dedifferentiated phenotype." Jat describes the derivation of conditionally immortal cell lines from a transgenic mouse and states (page 5096, right column, lines 4-6): "An additional problem associated with the introduction of immortalizing genes into cells is that these genes can alter normal cellular physiology..." As these references show, immortalized cells clearly differ from non-immortalized cells.

CONCLUSION

Applicants submit that the claims are in condition for allowance and such action is respectfully requested. Enclosed is a Petition to extend the period for replying to the Office Action for two months, to and including October 16, 2006.

If there are any additional charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: OCTOBER 16, 2006



James D. DeCamp
Reg. No. 43,580

Clark & Elbing LLP
101 Federal Street
Boston, MA 02110
Telephone: 617-428-0200
Facsimile: 617-428-7045

MICHAEL A. ROBINSON, Ph.D.
REG. NO. 57,903



NIH AIDS Research & Reference Reagent Program
20301 Century Boulevard
Bldg. 6, Suite 200
Germantown, MD 20874
USA

Phone: 240 686-4740
Fax: 301-515-4015
www.aidsreagent.org

DATA SHEET

Reagent: MT-4

Catalog Number: 120

Lot Number: 24 523466

Provided: 1.4×10^7 cells/vial and viability is 92%.

Cell Type: Human T cells isolated from a patient with adult T-cell leukemia.

Propagation Medium: RPMI 1640, 90%; fetal bovine serum, 10%.

Freeze Medium: RPMI 1640, 80%; fetal bovine serum, 20%; DMSO, 10%.

Sterility: Negative for mycoplasma, bacteria and fungi.

Growth Characteristics: Cells grow in suspension. Split cells 1:10 every three days. It may take a week before the cell viability increases above 50%.

Special Characteristics: HTLV-I transformed. Reverse transcriptase production is negative. p24 value is very low compared to the MT-2 cell line (an HTLV-I producer). Very useful for cytotoxicity inhibition assays for antiviral drugs. OKT4⁺, OKT4A⁺, and IL-2 receptor⁺.

Recommended Storage: Liquid nitrogen

Contributor: Dr. Douglas Richman

References:

Harada S, Koyanagi Y, Yamamoto N. Infection of HTLV-III/LAV in HTLV-I-carrying cells MT-2 and MT-4 and application in a plaque assay. *Science* **229**:563-566, 1985.

Larder BA, Darby G, Richman DD. HIV with reduced sensitivity to Zidovudine (AZT) isolated during prolonged therapy. *Science* **243**:1731-1734, 1989.

Pauwels R, De Clercq E, Desmyter J, Balzarini J, Goubau P, Herdewijn P, Vanderhaeghe H, Vandeputte M. Sensitive and rapid assay on MT-4 cells for detection of antiviral compounds against the AIDS virus. *J Virol Meth* **16**:171-185, 1987.

NOTE: Acknowledgment for publications should read "The following reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: MT-4 from Dr. Douglas Richman."

ALL RECIPIENTS OF THIS MATERIAL MUST COMPLY WITH ALL APPLICABLE BIOLOGICAL, CHEMICAL, AND/OR RADIOCHEMICAL SAFETY STANDARDS INCLUDING SPECIAL PRACTICES, EQUIPMENT, FACILITIES, AND REGULATIONS. NOT FOR USE IN HUMANS.

Exhibit 2



NIH AIDS Research & Reference Reagent Program
20301 Century Boulevard
Bldg. 6, Suite 200
Germantown, MD 20874
USA

Phone: 240 686-4740
Fax: 301-515-4015
www.aidsreagent.org

DATA SHEET

Reagent: Molt-4 Clone 8

Catalog Number: 175

Lot Number: 8 041241

Provided: 1 x 10⁷ cells/vial, viability=95%

Cell Type: Molt 4 was originally derived from the peripheral blood of a 19-year-old male with acute lymphoblastic leukemia in relapse. Clone 8 was obtained by subcloning in soft agarose by Kikukawa et al.

Propagation Medium: RPMI 1640, 90%; fetal bovine serum, 10%.

Growth Characteristics: Cells grow as a suspension. Maintain cells at 3 x 10⁵/ml. Split twice a week 1:4 to 1:5.

Sterility: Negative for mycoplasma, bacteria and fungi.

Special Characteristics: Highly susceptible to HTLV-III. Following infection, CPE is observed after one day, and giant cells after five days. Can be used to isolate and continuously produce SIV_{agm} virus (catalog #174).

Recommended Storage: Liquid nitrogen

Contributor: Dr. Ronald Desrosiers

References: Daniel MD, Li Y, Naidu YM, Durda PJ, Schmidt DK, Troup CD, Silva DP, MacKey JJ, Kestler HW III, Sehgal PK, King NW, Ohta Y, Hayami M, Desrosiers RC. Simian immunodeficiency virus from African green monkeys. *J Virol* **62**:4123-4128, 1988.

Kikukawa R, Koyanagi Y, Harada S, Kobayashi N, Hatanaka M, Yamamoto N. Differential susceptibility to the acquired immunodeficiency syndrome retrovirus in cloned cells of human leukemic T-cell line Molt-4. *J Virol* **57**:1159-1162, 1986.

NOTE: Acknowledgment for publications should read "The following reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Molt-4 Clone 8 from Dr. Ronald Desrosiers." Also include the references cited above in any publications.

Available only for non-commercial use. Requests from commercial organizations should be directed to Ronald C. Desrosiers, New England Regional Primate Research Center, Harvard Medical School, One Pine Hill Drive, Box 9102, Southborough, MA 01772-9102.

ALL RECIPIENTS OF THIS MATERIAL MUST COMPLY WITH ALL APPLICABLE BIOLOGICAL, CHEMICAL, AND/OR RADIOCHEMICAL SAFETY STANDARDS INCLUDING SPECIAL PRACTICES, EQUIPMENT, FACILITIES, AND REGULATIONS. NOT FOR USE IN HUMANS.

SV40-Immortalization of Rabbit Articular Chondrocytes: Alteration of Differentiated Functions

S. THENET,* P.D. BENYA, S. DEMIGNOT, J. FEUNTEUN, AND M. ADOLPHE

Laboratoire de Pharmacologie Cellulaire de l'École Pratique des Hautes Études, Centre de Recherches Biomédicales des Cordeliers, 15 rue de l'École de Médecine 75006 Paris (S.T., S.D., M.A.) and Laboratoire d'Oncologie Moléculaire, Institut Gustave Roussy, 94805 Villejuif (J.F.), France; Department of Orthopaedics, University of Southern California and Laboratory of Cartilage Cell Biology, Orthopaedic Hospital of Los Angeles, Los Angeles, California 90007-2697 (P.D.B.)

Cell lines were established from rabbit articular chondrocytes following transfection with a plasmid encoding SV40 early function genes. This resulted in cell immortalization (130 passages have been completed for the oldest cell line) with acquisition of characteristics of partial transformation such as reduced serum requirements for normal and clonal growth. The immortalized chondrocytes, called SVRAC, did not form multilayer foci when maintained in postconfluent culture. Their ability to form colonies in soft agar was not increased in comparison with normal chondrocytes, but they were weakly tumorigenic in nude mice. SVRAC lost the ability to synthesize type II collagen and Alcian blue-stainable matrix, which are markers of the differentiated chondrocyte phenotype, and synthesized predominantly type I collagen. Studies of collagen gene expression showed that $\text{pro}\alpha 1(\text{II})$ mRNA was undetectable, whereas $\text{pro}\alpha 1(\text{I})$ collagen mRNA was expressed even in late passage cultures. Unlike normal dedifferentiated chondrocytes, SVRAC were unable to re-express the differentiated phenotype in response to tridimensional culture or microfilament depolymerization. Cell lines obtained from chondrocytes transfected either in primary culture or just after release of cells from cartilage displayed the same behaviour. Thus SV40 early genes were able to immortalize rabbit articular chondrocytes, but the resulting cell lines displayed an apparently irreversibly dedifferentiated phenotype. These cell lines can be used as models to identify regulatory pathways that are required for the maintenance or reexpression of differentiated function in chondrocytes.

Chondrocytes are differentiated cells of mesenchymal origin that are responsible for the synthesis, maintenance, and degradation of cartilage. Investigation of their response to hormones, growth factors, and other environmental signals in health and disease is greatly hindered by their phenotypic instability in culture. We have immortalized chondrocytes in an attempt to stabilize the phenotype against culture-dependent modulation and have investigated the impact of immortalization on the mechanisms that mediate the normal phenotypic flexibility of these cells.

Pure and well-differentiated primary cultures are easily obtained (Green, 1971; Hough and Sokoloff, 1975) and produce a matrix of collagen and proteoglycan that is characteristic of cartilage. Type II collagen is generally used as a differentiation marker for chondrocytes, since it represents about 90% of the collagen synthesized by chondrocytes in cartilage or primary culture (Benya et al., 1978; Mayne and Von der Mark, 1983). Chondrocytes, serially subcultured as monolayers, progressively lose their differentiated phenotype, resulting in a change from the synthesis of type II collagen to a mixture of type I and type I trimer (Mayne et al., 1976; Benya et al., 1978). This transition can be

achieved more rapidly by viral transformation (Alema et al., 1985; Allebach et al., 1985), or by treatment with bromodeoxyuridine (Mayne et al., 1975; Saxe et al., 1985; Yasui et al., 1986), retinoic acid (Benya and Padilla, 1986; Yasui et al., 1986; Horton et al., 1987), or phorbol myristate acetate (PMA) (Finer et al., 1985). It is important that subcultured and retinoic acid-modulated chondrocytes can be induced to re-express the differentiated phenotype. Under the influence of serum-derived growth factors and a change to spherical shape in agarose gel culture, these cells regain high levels of type II collagen and proteoglycan synthesis (Benya and Shaffer, 1982). Microfilament modification appears to be the specific mediator of this process, since re-expression can also be induced without cell-rounding by treatment of monolayer cultures with the microfilament modifying drugs, cytochalasin B, and dihydrocytochalasin B (DHCB) (Benya and Shaffer, 1983; Benya et al., 1988).

Immortalization of primary cells has been accom-

Received May 14, 1991; accepted August 13, 1991.

*To whom reprint requests/correspondence should be addressed.

plished by infection with DNA tumor viruses or transfection with viral DNA fragments encoding Polyoma large T antigen (Rassoulzadegan et al., 1983), SV40 large T antigen (Petit et al., 1983), Adenovirus E1A (Ruley, 1983), and the Human Papilloma virus E7 region (Matlashewski et al., 1987). SV40 DNA has been widely used, although its effects on transformation, tumorigenicity, and differentiation vary considerably with the cell types and experimental conditions used (Monier, 1986).

Understanding the effect of immortalization on differentiation is important if cell lines are to be obtained that maintain their differentiated properties. Numerous studies have shown that SV40-immortalization of specialized cells results in the retention of at least some of their specific functions. This was the case with hepatocytes (Woodworth et al., 1986), renal tubular cells (Vandewalle et al., 1989), calvarial cells (Heath et al., 1989), uroepithelial cells (Christian et al., 1987), and neural cells (Evrard et al., 1990). Woodworth and Isom (1987) have emphasized the role of culture conditions during the process of immortalization and have shown that the use of a specialized chemically defined medium improved the expression of liver-specific functions by immortalized hepatocytes. In contrast, infection of rat pancreatic endocrine cells with SV40 resulted in the loss of insulin synthesis *in vitro*, but partial recovery of synthesis after passage *in vivo* (Niesor et al., 1979). Also, infection or transfection of keratinocytes with SV40 DNA apparently always resulted in an altered pattern of keratin expression (Steinberg and Defendi, 1979, 1983; Boukamp et al., 1988). Adipocyte differentiation has been reported to be blocked by expression of the SV40 virus (Tobe et al., 1987; Cherington et al., 1988). However, Estervig et al. (1990) have recently shown that stable transfection of SV40 early functions does not block adipocyte differentiation *per se*, but rather the prerequisite predifferentiation growth arrest. Conversely, transfection with adenovirus 5 E1A blocks expression of two myogenic regulatory factors (MyoD1 and myogenin) and consequently blocks differentiation in capable cells (Enkemann et al., 1990).

Immortalized chondrocyte cell lines have been obtained (Gionti et al., 1985; Horton et al., 1988), but none maintain all differentiated functions and responses to modulating signals. This may be due to the high lability of the chondrocyte phenotype. Since the effects of oncogene expression on division capacity may not necessarily prevent the transduction of environmental signals that lead to culture-dependent modulation, it is important to determine the phenotype of immortalized chondrocytes both before and after exposure to conditions that induce reexpression of the differentiated phenotype.

In this report, we demonstrate that immortalization of chondrocytes is possible with SV40 DNA but that this does not lead to stabilization of the phenotype. In addition, we demonstrate that several treatments, which induce re-expression of the differentiated phenotype in normal chondrocytes, fail to induce re-expression in SV40-immortalized chondrocytes. This suggests that immortalization in these cells does not merely permit or enhance the process of culture-dependent loss

of differentiated function, but may block re-expression signals directly or intervene at a higher regulatory level.

MATERIALS AND METHODS

Cell culture

Normal rabbit articular chondrocytes (RAC) were obtained from articular cartilage of 1–3-month-old rabbits (Fauve de Bourgogne) by enzymatic digestion using the technique of Green (1971). This technique gives a pure population of chondrocytes: fibroblast contamination of RAC cultures has never been observed under these conditions. The growth medium was Ham's F12 (Gibco-BRL) supplemented with 10% fetal calf serum (FCS) (IBF-Biotechnics, France) and Gentamicin (4 µg/ml), unless otherwise indicated.

Transfections

Transfection of normal RAC with the plasmid pAS encoding the early functions of SV40 (Benoist and Chambon, 1981) was performed using the calcium phosphate procedure (Graham and Van der Eb, 1973), at the 5th day of primary culture. Mock-transfected RAC received the plasmid pUC13. Cultures were passaged weekly and plated at 18,000 cells/cm². SV40-transfected RAC were selected by their ability to survive senescence, which inevitably occurred in normal RAC (Dominice et al., 1986) and mock-transfected RAC between passage 7 and passage 9. Immortalized chondrocytes were cloned and subcloned using cloning cylinders.

In other experiments, RAC were cotransfected just after release from cartilage with both pAS and the plasmid pSVtk-neo^R encoding the Neo^R gene (Nicolas and Berg, 1983). Selection in presence of G418-sulfate (Gibco-BRL) began 5 days after the transfection. Medium containing G418 (400 µg/ml) was changed three times a week. G418-resistant colonies appeared in approximately 3 weeks.

All established cell lines were passaged twice a week to avoid confluence and plated at 6,250 cells/cm².

Re-expression experiments

Cultures in agarose and in collagen gels were performed as previously described (Benya and Shaffer, 1982; Dewilde et al., 1988). Confluent cultures were treated for 10 days with 1×10^{-6} or 3×10^{-6} M DHCB (Sigma) in the presence of 10% FCS before collagen analysis.

Immunofluorescent staining for SV40 T-antigen

Cells were grown on glass coverslips for 4 days, washed in PBS, and fixed with a methanol/acetone mixture (30/70, v/v). Incubations with serum from a hamster bearing an SV40-induced tumor (diluted 1/50 in PBS), and then with Rhodamine-conjugated anti-hamster IgG (Cappel), (diluted 1/50 in PBS), were performed for 45 minutes in a humidified atmosphere at 37°C.

Determination of cell growth

Cells were plated in 35-mm culture dishes (7,000 cells/cm²). At the indicated times, cells were trypsinized and counted using an hemocytometer. SVRAC under-

went two passages in 5% or 2.5% FCS-Ham's F12 before growth curves were performed in these media in order to eliminate residual effects of culture in 10% FCS. For normal primary cultures, cells were seeded in 10% FCS-Ham's F12 to promote cell attachment, refed with the tested medium at day 2, harvested at confluence, and replated for growth experiments.

For determination of clonal growth, 100 cells were seeded into 60-mm dishes in 10%, 5%, or 2.5% FCS-medium after either two passages in these respective media for SVRAC or 3 days for normal RAC. Colonies were fixed and stained with May-Grunwald and Giemsa (Merck) after a 14-day incubation period. Cloning efficiencies were determined by counting macroscopic colonies (mean values from quadruplicate plates).

Colony formation in soft agar

The 10^5 cells suspended in Ham's F12 medium containing 0.3% agar (Difco Laboratories, Detroit, Michigan) and 10% FCS were seeded in 60-mm dishes previously coated with 0.6% agar in 10% FCS-medium. After an incubation period of 14 days, colonies were stained with neutral red (Fluka). Colonies of at least 10 viable cells were scored in five randomly selected fields (6 mm diameter) for each dish.

Tumorigenicity assay

On day 7 of culture, 10^6 SVRAC or normal RAC cells in 0.3 ml Ham's F12 were injected subcutaneously into female nude mice. Animals were examined weekly. Tumors were excised, fixed in Bouin's fluid, and processed for histological analysis.

Analysis of collagen gene expression

Collagen gene expression by SVRAC was characterized by polyacrylamide gel electrophoresis of biosynthetically labeled proteins and/or by Northern blotting with specific $\alpha 1(I)$ and $\alpha 1(II)$ procollagen cDNA probes, since both methods could not always be performed due to the particular culture conditions used for reexpression experiments.

Collagen synthesis

Duplicate cultures were labeled for 24 hours with L-(5- 3 H) proline (Amersham, 20–50 Ci/mmol, 50 μ Ci/ml) in DMEM containing 10% FCS supplemented with β -aminopropionitrile (62.5 μ g/ml, Sigma) and ascorbate (25 μ g/ml, Sigma). Whole cultures were adjusted to 0.5 M acetic acid and treated with 0.1 mg/ml of pepsin (Sigma) at 4°C for 24 hours. Pepsin was inactivated by the addition of TRIS to 120 mM and by titration to pH 7.4 with 10 N NaOH. NaCl was added to 0.5 M NaCl (final $[Na^+] = 1$ M) and cultures were extracted at 4°C for 24 hours. Carrier collagen in 1 M NaCl–50 mM TRIS, pH 7.5 was added to a concentration of 200–300 μ g/ml except for the experiment presented in Figure 3. Debris was removed by centrifugation, the mixture was adjusted to 4 M NaCl, left overnight at 4°C for collagen precipitation, and centrifuged for 1 hour at 30,000 g. The collagen pellet was rinsed twice with 40% ethanol and finally dissolved in 3 ml of 0.5N acetic acid. The samples were analysed by 7.5% SDS-PAGE under nonreducing conditions using a phastsystem (Pharmacia).

Purified radioactive collagen was lyophilized and cleaved under nitrogen with a fourfold (w/w) excess of cyanogen bromide (10 mg/ml in 70% formic acid) for 4 hours at 30°C, and treated again for 18 hours at room temperature. Samples were diluted with water and lyophilized twice before analysis on 10–15% SDS-polyacrylamide gels (Pharmacia) or by 2D-electrophoresis as previously described (Benay, 1981).

Gels were silver stained or processed for fluorography and exposed to Kodak XAR 5 film at -70°C .

RNA extractions and Northern analysis

Total RNA was extracted from cultured cells using guanidinium HCl solution according to Vaulont et al. (1984). From each sample, 20 μ g of RNA was denatured and fractionated on formaldehyde agarose gels. Equal loading of lanes was checked by examination of ribosomal RNA in the gel stained with ethidium bromide (0.5 μ g/ml). After transfer to "Positive" nylon filters (Appligène, France), UV photography of the gels and of the filters was performed to check the transfer efficiency.

The following cDNA clones for human procollagen mRNAs were used as hybridization probes: pHCallU (670bp) for the carboxy-propeptide domain of pro $\alpha 1(I)$ mRNAs (Mäkelä et al., 1988), and pHCar1 (585bp) for the carboxy-propeptide domain of pro $\alpha 1(II)$ mRNAs (Elima et al., 1985). The DNAs were labeled by random priming to a specific activity of approximately 1×10^9 cpm/ μ g.

Prehybridization was performed at 42°C for at least 3 hours in 50% deionized formamide, 5X SSC, 50 mM sodium phosphate pH 6.8, 1% SDS, 5X Denhardt's solution and 100 μ g/ml denatured salmon sperm DNA (Boehringer Mannheim). Hybridization was performed at 55°C in the same solution but in presence of 60% formamide for at least 14 hours. Filters were washed for 20 minutes with two changes of 2X SSC, 0.1% SDS at 55°C, then for 30 minutes with two changes of 0.1X SSC, 0.1% SDS at 55°C, and, finally, for 20 minutes in 0.1X SSC, 0.1% SDS at 65°C. These highly stringent conditions were chosen during preliminary studies that demonstrated no crosshybridization between the type II collagen probe and type I collagen mRNA (using mRNA from freshly isolated fibroblasts). However, some crosshybridization between the type I collagen probe and type II collagen mRNA was observed when the collagen II signal was very strong (see Figures 5, 8). Filters were then exposed at -70°C to X-ray films (Kodak-XAR-5) with intensifying screens. Dehybridizations were performed by washing the filters at 80°C in 1% SDS for 1–2 hours and controlled by autoradiography of the stripped filters.

RESULTS

Immortalization of rabbit articular chondrocytes

Rabbit articular chondrocytes (RAC) in primary monolayer culture (day 5) were transfected with the plasmids pAS, encoding SV40 large T and little t antigens (SVRAC), or pUC13 (mock-transfected RAC), and passaged weekly until passage 9 (P9). From first to ninth subculture, the two cell populations were morphologically indistinguishable and seemed to present all of characteristics of *in vitro* aging previously de-

scribed for normal RAC (Dominice et al., 1986). A decrease in cell proliferation rate and an increase in cell size were observed while cells became completely flattened and elongated. At P9, mock-transfected cells completely stopped dividing. Although medium was then changed twice a week for 2 more months, no proliferation could be observed. In contrast, cultures transfected with the plasmid pAS contained little clusters of actively proliferating polygonal cells at P9. At the next subculture (P10), most cells showed a polygonal shape and the cell number per flask markedly increased. These cells could be propagated further. Individual clonal cell lines were isolated by two successive cloning steps for subsequent characterization. They have undergone more than 130 passages and neither a decline in proliferation rate nor appearance of any characteristics of cell senescence have been observed.

Four other independent transfections with SV40 early function genes and the Neo gene were performed on spherical chondrocytes just after their release from cartilage. Immortalized cells were selected in presence of G418. One to three clones from each of the five transfections gave similar results when evaluated for immortalization, expression of the large T antigen, growth in soft agar, formation of multilayer foci, expression of $\alpha 1(I)$, and $\alpha 1(II)$ procollagen mRNAs and assays of reexpression. Tumorigenicity in nude mice was also similar for the three different clones that were tested. Therefore, results are presented for one of these clones, called SVRAC.

SVRAC, shown in phase contrast in Figure 1B, displayed the same polygonal morphology as normal chondrocytes (Fig. 1A). SV40 large T antigen, visualized by indirect immunofluorescent staining, was expressed in all SVRAC cells in exponential phase of growth (Fig. 1C). No fluorescence was detected in normal RAC.

Growth and transformation state of SV40-immortalized chondrocytes

Growth curves of normal RAC at the first passage (P1) and of SVRAC at P25 in standard culture conditions (10% FCS-medium in monolayer culture) are presented in Figure 2. Cell population doubling times were quite similar for normal and SVRAC (approximately 20 hours). The exponential phase was longer for immortalized cells resulting in a higher saturation density. Chondrocyte immortalization by SV40 early functions was accompanied by several characteristics of partial transformation: diminution of the serum requirement for growth or clonal growth (Table 1) and hypotetraploid karyotype with very few chromosome rearrangements (data not shown). Additional transformation criteria, such as formation of multilayer foci, growth in soft agar and tumorigenicity in nude mice have been defined for rodent fibroblast models (Benjamin, 1974). Since normal chondrocytes share some of these properties with transformed fibroblasts (Cossu et al., 1982), it was difficult to use these criteria to clearly estimate the transformation stage of SVRAC. Normal chondrocytes form colonies in 0.5% agarose as shown previously (Benya and Shaffer, 1982). We obtained similar colony forming efficiencies (approximately 0.2%) for normal chondrocytes and SVRAC

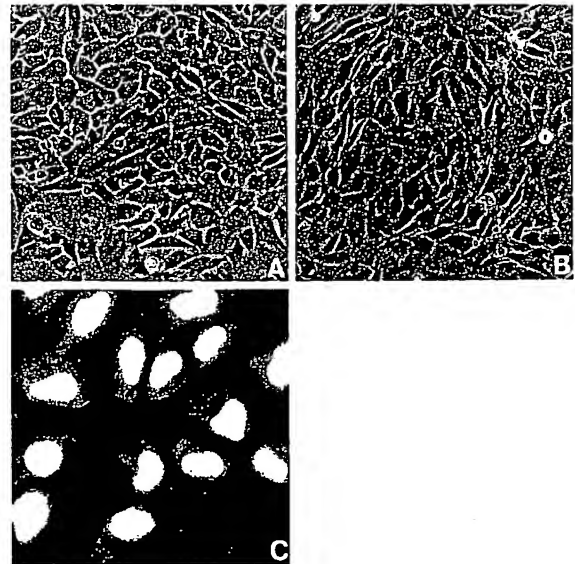


Fig. 1. Phase contrast photomicrographs of RAC primary culture at day 6 (A) and of SVRAC at passage 30 (B). Magnification: $\times 80$. Indirect immunofluorescent staining for SV40 large T antigen within the nuclei of SVRAC (C). Magnification: $\times 630$.

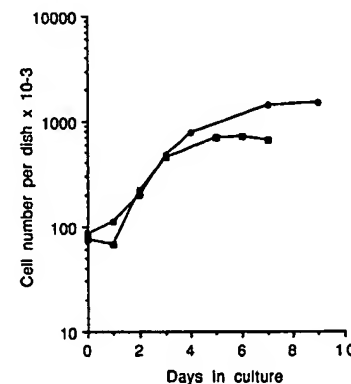


Fig. 2. Growth curves of normal RAC (■) and SVRAC (●). Cells were plated in 35 mm dishes in 2 ml Ham's F12 supplemented with 10% FCS. At the indicated times, three plates were trypsinized and counted in duplicate. Experimental error did not exceed 10%.

grown in 0.3% agar. In both cases, the colonies displayed similar sizes and morphologies and clearly differed from the much larger and very dense colonies obtained with a transformed hepatoma cell line under the same conditions (colony forming efficiency: 1.7%).

When 10^6 cells were injected into nude mice, the SVRAC cell line gave rise to tumors with the appearance of sarcoma in 4/5 animals after 3 months. These tumors grew slowly, however, and only reached a maximum diameter of 1–1.5 cm; none of these tumors expanded until it killed the host, as has been described

TABLE 1. Cell population doubling time and cloning efficiency of normal RAC and SVRAC in the presence of various concentrations of serum

FCS (%)	Cell population doubling time (h) ¹			Cloning efficiency (%) ²		
	10	5	2.5	10	5	2.5
RAC	17	38	47	27.5 ± 2.4	1.5 ± 1.3	1.7 ± 0.9
SVRAC	24	24	29	25.7 ± 4.6	25.5 ± 3.5	17 ± 5

¹Determined from the exponential phase of growth using curves plotted for each cell type and each serum concentration as described in Materials and Methods.

²10⁵ cells were seeded onto 60 mm dishes as described in Materials and Methods. Colonies were counted after 14 days incubation and staining with May-Grünwald and Giemsa (mean values from quadruplicate dishes ± SEM).

for *ras*-transformed cells (Land et al., 1983). When injected under the same conditions, normal primary RAC led to appearance of tumors within 2 months in 4/10 animals. However, only one of them gave rise to a cartilaginous nodule within 3 months, whereas the others were resorbed. The ability of normal chondrocytes to induce nodules in the nude mouse after subcutaneous inoculation has already been reported (Lipman et al., 1983; Takigawa et al., 1987).

When SVRAC were allowed to grow for 3 weeks without subculturing and with medium replacement twice a week, no multilayer foci were observed just as for normal RAC. Taken together, these results seem to suggest that SVRAC is not a fully transformed cell line.

Altered synthesis of cartilage specific products

Figure 3 presents a SDS-PAGE analysis of purified collagen extracted from SVRAC cells in monolayer culture. The SVRAC cell line only produced $\alpha 1$ collagen chains, no $\alpha 2$ chains were detected. However, SDS-PAGE analysis of intact collagen chains does not permit the separation of the $\alpha 1(I)$ chain of type I collagen and the $\alpha 1(II)$ chain of type II collagen; two-dimensional CNBr peptide maps of purified collagen was therefore performed for identification of the collagen type produced (Fig. 4). Chondrocytes in primary culture synthesized mostly type II collagen (Fig. 4A). In the SVRAC cell line, only type I collagen peptides could be identified. No trace of type II collagen CNBr peptides was detected (Fig. 4B). Since no $\alpha 2(I)$ chain was detected by SDS-PAGE analysis of intact collagen chains (Fig. 3) or in the 2D CNBr peptide map (Fig. 4B), the SVRAC cell line seems to produce type I trimer collagen rather than type I collagen.

A Northern blot analysis of total RNA from SVRAC cells (Fig. 5) revealed that $\alpha 1(II)$ procollagen mRNA was not detectable (Fig. 5A), suggesting that the suppression of the phenotype was controlled prior its translation. In contrast, SVRAC expressed $\alpha 1(I)$ procollagen mRNA as indicated by the characteristic doublet bands in Figure 5B, lanes 2 and 3. These bands were expressed with the same intensity at passage 30 and 80 and were not attenuated with subculture even at passage 130 (data not shown).

Proteoglycan deposition in cartilage tissue and chondrocyte culture can be measured by selective staining with Alcian blue (Capasso et al., 1982; Hassel and Horgan, 1982) and can also be used as marker of

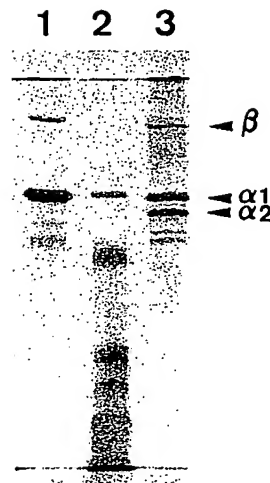


Fig. 3. 7.5% SDS-PAGE of pure type II collagen (Bioetica, France) (lane 1), collagen purified from SVRAC cells (lane 2), pure type I collagen (Bioetica, France) (lane 3). Proteins were silver stained. Positions of β , $\alpha 1$, and $\alpha 2$ chains are identified by arrowheads.

differentiated chondrocyte function. SVRAC staining with Alcian blue was 10- to 20-fold less than that observed in normal chondrocytes. This depended on seeding density and time in culture (data not shown).

In summary, the phenotype expressed by these SV40 immortalized chondrocytes showed the main characteristics of the "dedifferentiated" phenotypes already described for rabbit chondrocytes exposed to serial subculture (Benya et al., 1977, 1978) or treatment with retinoic acid (Benya and Padilla, 1986); namely, loss of type II collagen and cartilage proteoglycan synthesis and the predominant synthesis of type I collagen.

Assays of re-expression

As previously shown, the dedifferentiated phenotype obtained in normal chondrocytes by serial subculture is reversible (Benya and Shaffer, 1982, 1983; Benya et al., 1988). We investigated the capacity of SVRAC cells to reexpress type II collagen in a variety of culture conditions that have been described to induce re-expression by normal modulated chondrocytes.

SVRAC cells were cultured in gels of 0.5% low Tm agarose. The two-dimensional cyanogen bromide peptide map of the collagen isolated from SVRAC cultured in agarose for 10 days is presented in Figure 6A. The peptides were similar to those detected in confluent SVRAC monolayers (see Fig. 4B). Therefore, there was no detectable re-expression of type II collagen by SVRAC when cultured in agarose.

SVRAC cells were cultured in monolayer until confluency (7 days) and then maintained in postconfluent culture in the presence of DHCB (1 or 3×10^{-6} M) for 10 days. Collagens purified from control or DHCB-treated SVRAC were subjected to cyanogen bromide cleavage and fractionated on 10–15% SDS-PAGE gels



Fig. 4. Two-dimensional cyanogen bromide peptide maps of radioactive collagens purified from confluent normal RAC primary culture (A) and confluent SVRAC (B). A. The type II collagen peptides are identified by brackets. The positions of type I collagen peptides are indicated by parentheses. B. The type I collagen peptides are identified by parentheses. The positions of type II collagen peptides are indicated by brackets.

(Fig. 6B). No switching from type I to type II collagen synthesis was detected; $\alpha 1(\text{II})$ procollagen mRNA was not re-expressed following this treatment as shown by Northern blot analysis (Fig. 7A). Expression of $\alpha 1(\text{I})$ procollagen mRNA was not affected by DHCB treatment (Fig. 7B).

Culture of chondrocytes within collagen gels has been shown to permit maintenance of differentiated functions (Yasui et al., 1982; Dewilde et al., 1988; McClure et al., 1988) as well as chondrogenic differentiation of limb bud cells (Kimura et al., 1984). Figure 8 presents a Northern blot analysis of total RNA from normal first passage chondrocytes or SVRAC cultured either within collagen gels or in monolayer for 12 days. Normal first passage chondrocytes show a weak expression of mRNA for type II collagen compared to their collagen cultured counterparts (Fig. 8A), which display type II collagen mRNA at a level comparable to chondrocytes just released from cartilage. Thus these collagen culture conditions were adequate to cause reexpression of type II collagen synthesis in partially modulated normal chondrocytes. In contrast, SVRAC cultured within collagen gels for 12 days did not re-express type II collagen mRNA. Their type I collagen mRNA level was similar to the level in monolayer cultured SVRAC (Fig. 8B).

DISCUSSION

Rabbit articular chondrocytes in primary culture are generally considered to be a well-differentiated cell



Fig. 5. Northern blot analysis of pro $\alpha 1(\text{II})$ (A) and pro $\alpha 1(\text{I})$ collagen mRNA (B) in chondrocytes just released from cartilage (lane 1), SVRAC passage 30 (lane 2) and SVRAC passage 80 (lane 3). Ribosomal RNAs stained with ethidium bromide are presented in C to demonstrate equal loading of lanes. Positions of 28S and 18S rRNA are indicated by arrowheads. A signal using the probe for $\alpha 1(\text{I})$ collagen mRNA was not expected in freshly released chondrocytes (B, lane 1). Since a single band was revealed instead of the characteristic doublet always observed for $\alpha 1(\text{I})$ collagen mRNA, this signal is probably the result of cross hybridization between the $\alpha 1(\text{I})$ probe and $\alpha 1(\text{II})$ procollagen mRNA. This occurred in spite of the highly stringent conditions used for hybridization and washing (see Material and Methods) and was due to weak hybridization to the high level of type II collagen mRNA present in freshly released chondrocytes (A, lane 1).

population. They synthesize high levels of cartilage proteoglycans and type II collagen (Benya et al., 1978). In the present work, rabbit articular chondrocytes were transfected in primary culture with DNA from SV40 early genes. This led to immortalization of these cells and to an apparently irreversible suppression of their differentiated phenotype, characterized by complete loss of type II collagen synthesis, stable synthesis of type I collagen, and markedly reduced production of Alcian blue-stainable matrix. SVRAC have been passed more than 130 times, whereas the normal *in vitro* lifespan of RAC never exceeds nine subcultures (Dominice et al., 1986). SV40 large T antigen is likely to be responsible for immortalization since spontaneous escape from senescence by rabbit articular chondrocytes has never been reported and no proliferation was observed in mock-transfected cells, which were maintained for the same time in culture after they had reached senescence. Although the role of SV40 little t antigen is not yet well understood, we do not think that little t antigen is implicated in the process of immortalization *per se* because it has been shown that the large T antigen is sufficient to immortalize primary rodent fibroblasts (Petit et al., 1983). Moreover, transfection of chondrocytes with a plasmid encoding SV40 large T only (instead of large T and little t) gave rise to

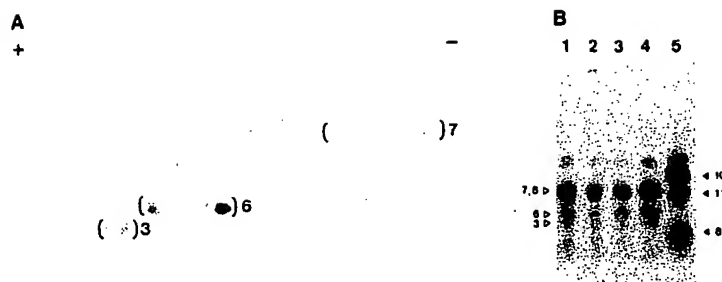


Fig. 6. A. 2D-CNBr peptide map of radioactive collagens purified from SVRAC cultured in agarose for 10 days. Type I collagen peptides are identified within parentheses. B. SDS-PAGE analysis of radioactive collagen CNBr peptides from confluent normal RAC primary culture (lane 5), confluent SVRAC (lane 4), 10 days postconfluent SVRAC (lane 3), postconfluent SVRAC treated for 10 days with 1×10^{-6} M DHCB (lane 2) or 3×10^{-6} M DHCB (lane 1). Type II collagen CNBr peptides are identified by closed arrowheads and type I collagen CNBr peptides by open arrowheads.

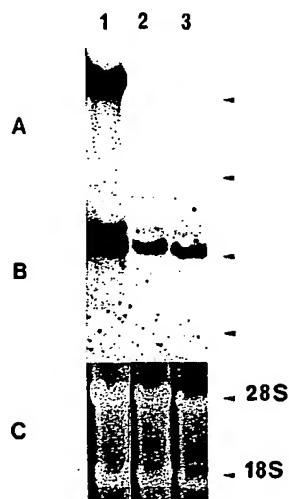


Fig. 7. Northern blot analysis of pro α 1(II) (A) and pro α 1(I) collagen mRNA (B) in normal RAC in primary culture (lane 1), SVRAC 10 days postconfluence (lane 2) and SVRAC treated for 10 days postconfluence with 3 μ M DHCB (lane 3). Ribosomal RNAs stained with ethidium bromide are presented in C. Positions of 28S and 18S rRNAs are indicated by arrowheads.

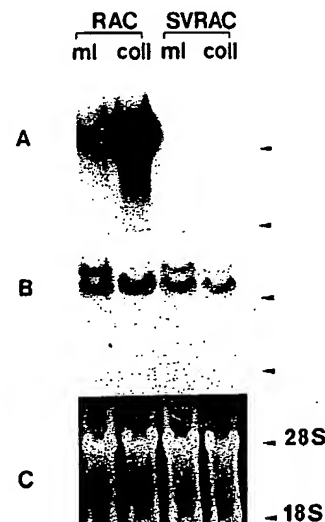


Fig. 8. Northern blot analysis of pro α 1(II) (A) and pro α 1(I) collagen mRNA (B) in normal RAC (first passage) and SVRAC cultured either in monolayer (ml) or within a collagen gel (coll). Ribosomal RNAs stained with ethidium bromide are presented in C. Positions of 28S and 18S rRNA are indicated by arrowheads. A single band was revealed for collagen cultured normal RAC mRNA hybridized with the pro α 1(I) collagen probe. This signal certainly results from the same cross hybridization phenomenon described in the legend of Figure 5.

immortalization with the same efficiency (data not shown).

SV40 infection of a variety of cell types, or transfection with SV40 DNA has often been reported to induce transformation as demonstrated by the ability to grow in semisolid medium and to induce tumors in nude mice or syngenic animals (Steinberg and Defendi, 1979,

1983; Heath et al., 1989; Vandewalle et al., 1989). Growth in soft agar and formation of multilayer foci are in vitro properties that are usually well correlated with tumorigenicity in animals, at least in fibroblasts mod-

els. The lack of correlation that we observed raises the question of validity of these criteria for some cellular models which differ from fibroblasts. The ability of normal chondrocytes to induce cartilaginous nodules in nude mice (Lipman et al., 1983; Takigawa et al., 1987) suggests that this property might not be really specific for transformed chondrocytes and decreases the significance of the fact that SVRAC were found to be tumorigenic in nude mice. SVRAC did not form multilayer foci, even when maintained for 3 weeks in post-confluent culture. They behaved in agarose like normal chondrocytes rather than transformed hepatoma cells. Hepatoma colonies exhibited the same morphology as those described by Alema et al. (1985) for chondroblasts transformed by the *v-src* oncogene following infection with the Rous Sarcoma Virus. In contrast, SVRAC colonies resembled those described for chondroblasts expressing the *v-myc* immortalizing oncogene after MC29 or HB1 infection (Alema et al., 1985). These results suggest that SVRAC were not fully transformed.

Chondrocyte phenotype lability in vitro has been the subject of numerous studies (see introductory section). Subculture, senescence, retinoic acid, BrdU, PMA, and retroviral infection have been shown to induce chondrocyte "dedifferentiation," which always results in a loss of type II collagen synthesis and production of other genetically distinct types of collagen (types I, I trimer, and III). The resulting phenotype depends on the modulating agent and the animal species. In all SV40 immortalized chondrocyte cell lines that we have examined, no trace of $\alpha 1(\text{II})$ procollagen mRNA could be detected and $\alpha 1(\text{I})$ procollagen mRNA was expressed. Intact chain analysis and two-dimensional cyanogen bromide peptide mapping of collagen synthesized by SVRAC showed that type I trimer was mainly expressed. Moreover, a substantial staining of the extracellular matrix by Alcian blue was never obtained. Despite these similarities to other modulated chondrocytes, SVRAC differed dramatically in their capacity to re-express the differentiated phenotype in response to tridimensional culture or microfilament modification by DHCB. These conditions, which were effective in subcultured and retinoic acid modulated chondrocytes, did not alter the phenotype of any of the immortalized cell lines. Thus expression of SV40 DNA has blocked the microfilament/growth factor pathway of reexpression or activated a modulation mechanism not previously identified.

Several hypothesis concerning the cause of phenotypic modulation in chondrocytes can be considered. First, the effects on chondrocyte differentiation could be the consequence of stimulated proliferation, since in numerous cellular models, proliferation is incompatible with terminal differentiation. It does not seem, however, that active proliferation is totally incompatible with chondrocyte differentiation. TGF β in the presence of FGF was reported to exert a positive effect on chondrocyte proliferation while stimulating glycosaminoglycan and type II collagen synthesis (Hiraki et al., 1988; Iwamoto et al., 1989). Moreover, in both reports of immortalization of chondrocytes by the *v-myc* oncogene, the cell lines showed higher proliferation rates than primary chondrocytes while maintaining at

least some differentiated chondrocyte properties (Gionti et al., 1985; Horton et al., 1988). The effects of oncogenes from DNA tumor viruses such as SV40, Polyoma, or Adenovirus, on chondrocyte proliferation and differentiation have never been studied to our knowledge. However, it has been shown that SV40 T-antigen was able to block the adipocyte differentiation of a 3T3T murine mesenchymal stem line by decreasing its ability to undergo predifferentiation proliferation arrest. If predifferentiation growth arrest was accomplished using stringent differentiation-inducing conditions, terminal adipocyte differentiation occurred, suggesting that SV40 did not block the process of differentiation per se in this cellular system (Estervig et al., 1990). On the contrary, in our model, none of the culture conditions that can be used to make modulated chondrocytes re-express the differentiated phenotype was able to reinduce type II collagen synthesis by SVRAC. These results were obtained even though proliferation was not favoured in these conditions: DHCB treatment is performed after confluence; proliferation is markedly slowed down in agarose gels (Benya and Shaffer, 1982), as well as in collagen gels (Dewilde et al., 1988). Thus conditions that combine low proliferation and shape modifications are not sufficient to reinduce differentiation in SVRAC.

As a second hypothesis, modulation of the phenotype could be directly triggered by expression of SV40 early functions. The effects of expression of several retroviral oncogenes on chondrocyte differentiation have been studied in avian models. It has been shown that infection of chondroblasts with the retrovirus MC29 or HB1 (expressing *v-myc*) did not trigger any important modification of the expression of cartilage specific products, i.e., type II collagen, core protein, and cartilage proteoglycans. On the contrary, infection with RSV (expressing *src*) or MH2 (expressing *myc* + *mil*) resulted in a substantial reduction of type II collagen, core protein, and cartilage proteoglycan synthesis, and even in the suppression of type I collagen synthesis in the case of MH2 (Alema et al., 1985). *Src* seems to exert its effects on chondrocyte differentiation independently of its effects on cell proliferation, since in chondroblasts infected with a temperature sensitive transformation mutant of RSV, shifts between permissive and non-permissive temperatures resulted in shifts in sulfated proteoglycan synthesis without corresponding effects on cell growth rates (Pacifci et al., 1977). The effects of SV40 early functions on chondrocyte differentiation that were observed in this work resemble those obtained with *src* in the avian model: loss of type II collagen and cartilage proteoglycan synthesis but maintenance of type I collagen synthesis. Moreover, the loss of response to suspension culture (i.e., shape modification) was a characteristic observed in *src* (Boettiger and Menko, 1988) but not in *myc* transformed chondroblasts (Horton et al., 1988).

The mechanism by which SV40 early functions modulate the differentiated chondrocyte phenotype remains to be elucidated. However, the fact that SV40 large T antigen has been shown to induce or activate a serine/threonine kinase (Scheidtmann and Haber, 1990) suggests one possible mechanism: activation of protein kinase C in SV40 immortalized chondrocytes.

This possibility is indirectly supported by the observation that phorbol-12-myristate-13-acetate (PMA), which is a potent tumor promotor and an activator of protein kinase C, causes chondrocyte dedifferentiation (Finer et al., 1985). In addition, an inhibitor of protein kinase C, staurosporine, enhances reexpression of the differentiated chondrocyte phenotype (Benya and Padilla, 1990). Rat fibroblast cell lines that overproduce the $\beta 1$ isoform of protein kinase C were shown to display several abnormalities in growth control: they reached higher saturation density, they were weakly tumorigenic in nude mice (Housey et al., 1988), and were much more sensitive to low concentrations of serum than the control cell lines (Hoshina et al., 1990). All of these properties were observed in SV40 immortalized chondrocytes.

The data presented here cannot exclude the possibility that modulation of the phenotype in SVRAC simply resulted from long-term monolayer culture. The earliest reexpression assays that we performed were at passage 10–15, depending on which cell line was tested. Benya and Shaffer have shown that the time required for elevated collagen II re-expression by normal chondrocytes in agarose gel culture increases with the subculture number in monolayer (1982). However, qualitative reexpression of the differentiated phenotype was always essentially complete by 10 days in agarose even for 6th subcultures. Moreover, this hypothesis does not seem valid since it has been shown that quail chondrocytes could be immortalized after MC29 infection and retained the ability to synthesize type II collagen and other cartilage specific markers, even after 70 passages in culture (Gionti et al., 1985). Rat chondrocytes immortalized by the *myc* oncogene, using a recombinant retrovirus, synthesized Alcian blue stainable matrix, core proteins, and link proteins. Although $\alpha 1(\text{II})$ collagen mRNA production was only 1% of the level observed in primary rat chondrocytes, these cells were still able to modulate collagen I expression in response to suspension culture (Horton et al., 1988).

Although expression of SV40 DNA has led to chondrocyte immortalization without stabilizing the differentiated phenotype, it has focused attention on the normal mechanisms that control phenotypic expression in chondrocytes. Large T antigen may block re-expression of differentiated functions in SV40-immortalized chondrocytes by interfering with usually effective signals derived from alteration in microfilament structures or growth factor receptors. Alternatively, large T antigen may cause constitutive activation of the protein kinase that is involved in normal modulation (Benya and Padilla, 1990) or activate a different kinase with equivalent results. Finally large T antigen may inhibit the expression of regulatory genes that determine the chondrocyte phenotype, as has been shown for the E1A-dependent suppression of MyoD and myogenin in myogenic cells (Enkemann et al., 1990). The availability of SVRAC will enhance our ability to identify and verify such chondrocyte regulatory pathways.

ACKNOWLEDGMENTS

We are very grateful to Dr. E. Frindel, Mr. P. Ardouin, and Mrs. G. Bretou for their help in the nude

mice studies, and to Dr. B. Dutrillaux for karyotypic analysis. We thank Dr. X. Ronot, Dr. B. Gaillard-Froger, and Dr. L. Mir for their help at the beginning of this work, and Dr. C. Gespach, Dr. P. Rouget, and their research teams for their constant interest and encouragement. This work was supported by grants from the Ministère de la Recherche et de la Technologie (87T0422) and from INSERM (897001). S.T. was supported by a fellowship from the Association pour la Recherche sur le Cancer. P.D.B. was supported by a National Institutes of Health grant, AM16404, and the Cora Kaiser Fund of Orthopaedic Hospital.

LITERATURE CITED

- Alema, S., Tato, F., and Boettiger, D. (1985) *Myc* and *src* oncogenes have complementary effects on cell proliferation and expression of specific extracellular matrix components in definitive chondroblasts. *Mol. Cell. Biol.*, 5:538–544.
- Allebach, E.S., Boettiger, D., Pacifici, M., and Adams, S.L. (1985) Control of types I and II collagen and fibronectin gene expression in chondrocytes delineated by viral transformation. *Mol. Cell. Biol.*, 5:1002–1008.
- Benjamin, T.L. (1974) Methods in cell transformation by tumor viruses. In: *Methods in Cell Biology*, Vol. VIII. J.M. Prescott, ed. Academic Press, New York, pp. 367–437.
- Benoist, C., and Chambon, P. (1981) In vivo sequence requirements of the SV40 early promoter region. *Nature (London)*, 290:304–310.
- Benya, P.D. (1981) Two dimensional CNBr peptide patterns of collagen types I, II and III. *Collagen Rel. Res.*, 1:17–26.
- Benya, P.D., and Shaffer, J.D. (1982) Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell*, 30:215–224.
- Benya, P.D., and Shaffer, J.D. (1983) Microfilament involvement in the reexpression of the differentiated chondrocyte phenotype. *Orthop. Trans.*, 7:263.
- Benya, P.D., and Padilla, S.R. (1986) Modulation of the rabbit chondrocyte phenotype by retinoic acid terminates type II collagen synthesis without inducing type I collagen: the modulated phenotype differs from that produced by subculture. *Develop. Biol.*, 118:298–305.
- Benya, P.D., and Padilla, S.R. (1990) Staurosporine, an inhibitor of protein kinase C, enhances reexpression of the differentiated chondrocyte collagen phenotype. *Trans. Orthop. Res. Soc.*, 15:183.
- Benya, P.D., Padilla, S.R., and Nimni, M.E. (1977) The progeny of rabbit articular chondrocytes synthesize collagen types I and III and type I trimer but not type II. Verifications by cyanogen bromide peptide analysis. *Biochemistry*, 16:865–872.
- Benya, P.D., Padilla, S.R., and Nimni, M.E. (1978) Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. *Cell*, 15:1313–1321.
- Benya, P.D., Brown, P.D., and Padilla, S.R. (1988) Microfilament modification by dihydrocytochalasin B causes retinoic acid-modulated chondrocytes to reexpress the differentiated phenotype without a change in cell shape. *J. Cell. Biol.*, 106:161–170.
- Boettiger, D., and Menko, S.A. (1988) Effects of oncogenes on chondrogenesis. *Pathol. Immunopathol. Res.*, 7:32–38.
- Boukamp, P., Petrusseuska, R.T., Breitkreuz, D., J. Hornung, A. Markham, and N. Fusenig (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.*, 106:761–771.
- Capasso, O., Gionti, E., Pontarelli, G., Ambesi-Impombato, F.S., Nitsch, L., Tajana, G., and Cancedda, R. (1982) The culture of chick embryo chondrocytes and the control of their differentiated functions. *Exp. Cell Res.*, 142:197–206.
- Cheriton, V., Brown, M., Paucha, E., St-Louis, J., Spiegelman, B.M., and Roberts, T.M. (1988) Separation of Simian Virus 40 large T-antigen transforming and origin-binding functions from the ability to block differentiation. *Mol. Cell. Biol.*, 8:1380–1384.
- Christian, B.J., Loretz, L.J., Oberley, T.D., and Reznikoff, C.A. (1987) Characterization of human uroepithelial cells immortalized in vitro by Simian Virus 40. *Cancer Res.*, 47:6066–6074.
- Cossu, G., Warren, L., Boettiger, D., Holtzer, H., and Pacifici, M. (1982) Similar glycopeptides in normal chondroblasts and in Rous Sarcoma Virus-transformed fibroblasts. *J. Biol. Chem.*, 257:4463–4468.
- Dewilde, B., Benel, L., Hartmann, D.-J. and Adolphe, M. (1988)

- Subculture of rabbit articular chondrocytes within a collagen gel: Growth and analysis of differentiation. *Cytotechnology*, 1:123-132.
- Dominic, J., Levasseur, C., Laro, S., Ronot, X., and Adolphe, M. (1986) Age-related changes in rabbit articular chondrocytes. *Mech. Ageing Dev.*, 37:231-240.
- Elima, K., Mäkelä, J.K., Vuorio, T., Kauppinen, S., Knowes, J., and Vuorio, E. (1985) Construction and identification of a cDNA clone for human type II procollagen mRNA. *Biochem. J.*, 229:183-188.
- Enkemann, S.A., Konieczny, S.F., and Taparowsky, E.J. (1990) Adenovirus 5 E1A represses muscle-specific enhancers and inhibits expression of the myogenic regulatory factors genes, MyoD1 and myogenin. *Cell Growth Differ.*, 1:375-382.
- Estévez, D.N., Mnoo, P., Tzen, C.-Y., and Scott, R.E. (1990) Three distinct effects of SV40 T-antigen gene on cellular differentiation. *J. Cell. Physiol.*, 142:552-558.
- Evvard, C., Borde, I., Marin, P., Galiana, E., Prémont, J., Gros, F., and Rouget, P. (1990) Immortalization of bipotential and plastic glioneuronal precursor cells. *Proc. Natl. Acad. Sci. U.S.A.*, 87:3062-3066.
- Finer, M., Gerstenfeld, L., Doty, P., and Boedtker, H. (1985) Collagen expression in embryonic chicken chondrocytes treated with phorbol myristate acetate. *Mol. Cell. Biol.*, 5:1415-1424.
- Gionti, E., Pontarelli, G., and Cancedda, R. (1985) Avian myelocytomatosis virus immortalizes differentiated quail chondrocytes. *Proc. Natl. Acad. Sci. U.S.A.*, 82:2756-2760.
- Graham, F.L. and Van der Eb, A.J. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology*, 52:456-467.
- Green, W.T. (1971) Behavior of articular chondrocytes in culture. *Clin. Orthop.*, 75:248-260.
- Hassel, J.R., and Horgan, E.A. (1982) Chondrogenesis: a model developmental system for measuring the teratogenic potential of compounds. *Teratog. Carcinog. Mutagen.*, 2:325-331.
- Heath, J.K., Rodan, S.B., Yoon, K., and Rodan, G.A. (1989) Rat calvarial cell lines immortalized with SV40 large T antigen: constitutive and retinoic acid-inducible expression of osteoblastic features. *Endocrinology*, 124:3060-3068.
- Hiraki, Y., Inoue, H., Hirai, R., Kato, Y., and Suzuki, F. (1988) Effect of transforming growth factor β on cell proliferation and glycosaminoglycan synthesis by rabbit growth plate chondrocytes in culture. *Biochim. Biophys. Acta*, 969:91-99.
- Horton, W.E., Yamada, Y., and Hassel, J.R. (1987) Retinoic acid rapidly reduces cartilage matrix synthesis by altering gene transcription in chondrocytes. *Develop. Biol.*, 123:508-516.
- Horton, W.E., Cleveland, J., Rapp, U., Nemuth, G., Bolander, M., Doege, K., Yamada, Y., and Hassel, J.R. (1988) An established rat cell line expressing chondrocyte properties. *Exp. Cell Res.*, 178:457-468.
- Hoshina, S., Ueffing, M., and Weinstein, I.B. (1990) Growth factor-induced DNA synthesis in cells that overproduce protein kinase C. *J. Cell. Physiol.*, 145:262-267.
- Hough, A.J., and Sokoloff, L. (1975) Tissue sampling as a potential source of error in experimental studies of cartilage. *Connect. Tiss. Res.*, 3:27-31.
- Housey, G.M., Johnson, M.D., Hsiao, W.-L.W., O'Brian, C.A., Murphy, J.M., Kirschmeier, P., and Weinstein, I.B. (1988) Overproduction of protein kinase C causes disordered growth in rat fibroblasts. *Cell*, 52:343-354.
- Iwamoto, M., Sato, K., Nakashima, H., Fuchihata, H., Suzuki, F., and Kato, Y. (1989) Regulation of colony formation of differentiated chondrocytes in soft agar by transforming growth factor β . *Biochem. Biophys. Res. Commun.*, 159:1006-1011.
- Kimura, T., Yasui, N., Ohsawa, S., and Ono, K. (1984) Chondrogenic differentiation of limb bud cells in collagen gel culture. *Biomedical Res.*, 5:465-473.
- Land, H., Parada, L.F., and Weinberg, R.A. (1983) Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature*, 304:596-602.
- Lipman, J.M., Mc Devitt, C.A., and Sokoloff, L. (1983) Xenografts of articular chondrocytes in the nude mouse. *Calcif. Tissue Int.*, 35:767-772.
- Mäkelä, J.K., Raassina, M., Virta, A., and Vuorio, E. (1988) Human procollagen (I) collagen: cDNA sequence for the C-propeptide domain. *Nucl. Acid. Res.*, 16:349.
- Matlashewski, G., Schneider, J., Banks, L., Jones, N., Murray, A., and Crawford, L. (1987) Human papilloma virus type 16 DNA cooperates with activated ras in transforming primary cells. *EMBO J.*, 6:1741-1746.
- Mayne, R., Vail, M.S., and Miller, E.J. (1975) Analysis of changes in collagen biosynthesis that occur when chick chondrocytes are grown in 5-bromo-2'-deoxyuridine. *Proc. Natl. Acad. Sci. U.S.A.*, 72:4511-4515.
- Mayne, R., Vail, M.S., Mayne, P.M., and Miller E.J. (1976) Changes in type of collagen synthesized as clones of chick chondrocytes grow and eventually lose division capacity. *Proc. Natl. Acad. Sci. U.S.A.*, 73:1674-1678.
- Mayne, R., and Von der Mark, K. (1983) in: *Cartilage*, Vol. 1. B.K. Hall, ed. Academic Press, New York, pp. 181-214.
- McClure, J., Bates, G.P., Rowston, H., and Grant, M.E. (1988) A comparison of the morphological, histochemical and biochemical features of embryonic chick sternal chondrocytes in vivo with chondrocytes cultured in three-dimensional collagen gels. *Bone Miner.*, 3:235-247.
- Monier, R. (1986) Transformation by SV40 and Polyoma. In: *The Papovaviridae*, Vol. 1. N.P. Salzman, ed. Plenum Press, New York, pp. 247-294.
- Nicolas, J.-F., and Berg, P. (1983) Regulation of expression of genes transduced into embryonal carcinoma cells. *Cold Spring Harbor Conf. Cell Proliferation*, 10:469-485.
- Niesor, E.J., Wollheim, C.B., Mintz, D.H., Blondel, B., Renold, A.E., and Weil, R. (1979) Establishment of rat pancreatic endocrine cell lines by infection with Simian Virus 40. *Biochem. J.*, 178:559-568.
- Pacifici, M., Boettiger, D., Roby, K., and Holtzer, H. (1977) Transformation of chondroblasts by Rous Sarcoma Virus and synthesis of the sulfated proteoglycan matrix. *Cell*, 11:891-899.
- Petit, C.A., Gardes, M., and Feunteun, J. (1983) Immortalization of rodent embryo fibroblasts by SV40 is maintained by the A gene. *Virology*, 127:74-82.
- Rassoulzadegan, M., Naghashfar, Z., Cowie, A., Carr, A., Grisoni, M., Kamen, R., and Cuzin, F. (1983) Expression of the large T protein of polyoma virus promotes the establishment in culture of "normal" rodent fibroblasts cell lines. *Proc. Natl. Acad. Sci. U.S.A.*, 80:4354-4358.
- Ruley, H.E. (1983) Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. *Nature (London)*, 304:602-606.
- Saxe, S.A., Lukens, L.N., and Pawlowski, P.J. (1985) Changes in the nuclear and cytoplasmic levels of type I and type II collagen RNAs during growth of chondrocytes in 5-bromo-2'-deoxyuridine. *J. Biol. Chem.*, 260:3812-3819.
- Scheidtmann, K.H., and Haber, A. (1990) Simian virus 40 large T antigen induces or activates a protein kinase which phosphorylates the transformation-associated protein p53. *J. Virol.*, 64:672-679.
- Steinberg, M.L. and Defendi, V. (1979) Altered pattern of growth and differentiation in human keratinocytes infected by simian virus 40. *Proc. Natl. Acad. Sci. U.S.A.*, 76:801-805.
- Steinberg, M.L., and Defendi, V. (1983) Transformation and immortalization of human keratinocytes by SV40. *J. Invest. Dermatol.*, 81:131-136.
- Tagigawa, M., Shirai, E., Fukuo, K., Tajima, K., Mori, Y., and Suzuki, F. (1987) Chondrocytes dedifferentiated by serial monolayer culture form cartilage nodules in nude mice. *Bone Miner.*, 2:449-462.
- Tobe, K., Kasuga, M., Kitasato, H., Takaku, F., Takano, T., and Segawa, K. (1987) Differential effects of DNA tumor virus nuclear oncogene products on adipocyte differentiation. *FEBS Lett.*, 215:345-349.
- Vandewalle, A., Lelongt, B., Geniteau-Legendre, M., Baudouin, B., Antoine, M., Estrade, S., Chatelet, F., Verroust, P., Cassingena, R., and Ronco, P. (1989) Maintenance of proximal and distal cell functions in SV40-transformed tubular cell lines derived from rabbit kidney cortex. *J. Cell. Physiol.*, 141:203-221.
- Vaulont, S., Munnich, A., Marie, J., Reach, G., Pichard, A.-L., Simon, M.-P., Besmond, C., Barbry, P., and Kahn, A. (1984) Cyclic AMP as a transcriptional inhibitor of upper eukaryotic gene transcription. *Biochem. Biophys. Res. Com.*, 125:135-141.
- Woodworth, C.D., Secott, T., and Isom, H.C. (1986) Transformation of rat hepatocytes by transfection with SV40 DNA to yield proliferating differentiated cells. *Cancer Res.*, 46:4018-4026.
- Woodworth, C.D., and Isom, H.C. (1987) Regulation of albumin gene expression in a series of rat hepatocyte cell lines immortalized by simian virus 40 and maintained in chemically defined medium. *Mol. Cell. Biol.*, 7:3740-3748.
- Yasui, N., Ohsawa, S., Ochi, T., Nakashima, H., and Ono, K. (1982) Primary culture of chondrocytes embedded in collagen gels. *Exp. Cell Res.*, 50:92-100.
- Yasui, N., Benya, P.D., and Nimni, M.E. (1986) Coordinate regulation of type IX and type II collagen synthesis during growth of chick chondrocytes in retinoic acid or 5'-Bromo-2'-deoxyuridine. *J. Biol. Chem.*, 261:7997-8001.

Direct derivation of conditionally immortal cell lines from an *H-2K^b*-tsA58 transgenic mouse

PARMJIT S. JAT^{*§}, MARK D. NOBLE^{*}, PARIS ATALIOTIS^{*}, YUJIRO TANAKA[†], NIKOS YANNOUSOS[†], LENA LARSEN[‡], AND DIMITRIS KIOUSSIS[†]

^{*}Ludwig Institute for Cancer Research and [†]Human Tumour Immunology Group, Imperial Cancer Research Fund, Courtauld Building, 91 Riding House Street, London W1P 8BT, United Kingdom; and [‡]Laboratory of Gene Structure and Expression, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom

Communicated by Avron Mitchison, January 24, 1991

ABSTRACT Studies on cell lines have greatly improved our understanding of many important biological questions. Generation of cell lines is facilitated by the introduction of immortalizing oncogenes into cell types of interest. One gene known to immortalize many different cell types *in vitro* encodes the simian virus 40 (SV40) large tumor (T) antigen (TAg). To circumvent the need for gene insertion *in vitro* to generate cell lines, we created transgenic mice harboring the SV40 TAg gene. Since previous studies have shown that TAg expression in transgenic mice is associated with tumorigenesis and aberrant development, we utilized a thermolabile TAg [from a SV40 strain, tsA58, temperature sensitive (ts) for transformation] to reduce the levels of functional TAg present *in vivo*. To direct expression to a broad range of tissues, we used the mouse major histocompatibility complex *H-2K^b* promoter, which is both widely active and can be further induced by interferons. tsA58 TAg mRNA was expressed in tissues of all animals harboring the hybrid construct. Development of all tissues was macroscopically normal except for thymus, which consistently showed hyperplasia. Fibroblast and cytokeratin⁺ thymic epithelial cultures from these mice were readily established without undergoing crisis and were conditionally immortal in their growth; the degree of conditionality was correlated with the levels of tsA58 TAg detected. One strain of *H-2K^b*-tsA58 mice has been bred through several generations to homozygosity and transmits a functional copy of the transgene.

Although the use of cell lines has been of central importance in the development of cellular and molecular biology, the limited number of available cell lines and the difficulty in obtaining new ones have impeded many areas of study. The increasing realization of the value of cell lines has been associated with a continual evolution in relevant technologies. Initially, cell lines were obtained only as tumor cells or as spontaneously immortalized variants of cells that grew readily in tissue culture (1). More recently, transfection and retroviral-mediated gene insertion of immortalizing genes have been used to facilitate the production of cell lines from various tissues (2–11). However, transfection requires a large number of target cells to ensure that some cells of interest stably integrate the chosen DNA in a position suitable for expression. Viral-mediated gene transfer can be carried out with fewer cells by cocultivation of target cells with virus-producing feeder layers; however, this method still requires that target cells are dividing to achieve integration of the selected DNA into the genome (2). Moreover, both of these technologies require the growth of cells for extended periods of time in culture, under selective pressure, to obtain sufficient numbers of cells expressing the immortalizing gene to allow experimentation. In addition, lines from putatively

identical cells have different sites of gene integration and often express markedly different behaviors and levels of expression of the immortalizing gene.

An additional problem associated with the introduction of immortalizing genes into cells is that these genes can alter normal cellular physiology (1, 12), a problem that is also relevant to the isolation of cell lines from transgenic animals (e.g., refs. 28–30, 34, 35). This problem theoretically can be overcome through the use of conditional immortalizing genes, which allow the generation of continuously proliferating cell lines capable of differentiation after inactivation of the immortalizing gene. For example, the simian virus 40 (SV40) mutant temperature-sensitive (ts) strain tsA58, which encodes a thermolabile large tumor (T) antigen (TAg) capable of immortalization only at the permissive temperatures, has been used in the generation of a variety of conditionally immortal cell lines (13–17). However, introduction of conditional immortalizing genes *in vitro* still suffers from the problems discussed above for transfection and infection of wild-type genes.

To overcome some of the difficulties in the generation of cell lines, an approach was developed that facilitates and ensures the presence of a conditional oncogene in all of the cells of interest at a common integration site. Thus, transgenic mice were generated that harbor SV40 strain tsA58 early region coding sequences under the control of the mouse major histocompatibility complex *H-2K^b* class I promoter (18–21). This promoter is active at various levels in different tissues of the body but can be induced to higher levels of expression in almost all cells by exposure of the cells to interferons (IFNs) (21–23). Skin fibroblast cultures derived from these mice were conditional in their growth, as has been demonstrated for rat embryo fibroblasts immortalized by infection with a recombinant retrovirus that transduces the tsA58 TAg (15). Work with transfection and viral-mediated gene insertion has consistently indicated that techniques developed through the use of fibroblast populations can be transferred readily to other cell systems. This is also the case with the cells obtained from these transgenic mice, and cytokeratin⁺ thymic epithelial cell lines that were also established readily from these animals.

MATERIALS AND METHODS

Construction of the Transgene. The 5' flanking promoter sequences and the transcriptional initiation site of the mouse *H-2K^b* class I gene were fused to the SV40 tsA58 early region coding sequences. The 4.2-kilobase (kb) *EcoRI*-*Nru* I fragment encompassing the *H-2K^b* promoter sequences was ligated to the 2.7-kb *Bgl* I-*Bam* HI fragment derived from the tsA58 early region gene and pUC19 double-digested with

EcoRI and *BamHI*. The *Bgl* I site was blunted by using the Klenow fragment of *Escherichia coli* DNA polymerase I to allow fusion to the *Nru* I site. For microinjection, the *H-2K^b-tsA58* DNA fragment was isolated free of vector sequences by digestion with *EcoRI* and *Sal* I (24). All DNA manipulations were carried out by standard procedures (25).

RNA Blot-Hybridization (Northern) Analysis. RNA was prepared and analyzed by hybridization to a ³²P-labeled SV40 early region fragment using standard procedures (25, 26).

Cloning and Proliferation Assays. Skin fibroblasts were prepared as described (27) and grown in Dulbecco's modified Eagle's medium supplemented with 100 units of penicillin, streptomycin, and recombinant murine γ interferon (IFN- γ , Genzyme) per ml. For colony assays, 10³ cells derived from cultures grown at 33°C in the presence of IFN- γ were replated in 6-cm tissue culture dishes in the absence of IFN- γ at 33°C to allow adherence under identical conditions. Growth conditions were changed after 24 hr to indicated conditions (Fig. 2). Cultures were refed twice weekly for 14 days and stained with 2% methylene blue; colonies then were counted blind. For proliferation assays, 10⁴ cells were similarly plated, and dishes were analyzed after 7 and 14 days. A single dish was also counted on day 1 to determine the number of adhering cells. All determinations were carried out in duplicate.

Immunoblot (Western Blot) Analysis. Preparation of protein extracts and their analysis with mAb PAb419, directed against TAG, were performed by standard procedures (26).

Immunofluorescence Analysis of Thymic Epithelial Cells. Cells grown on poly(L-lysine)-coated coverslips were stained with an antibody specific for keratin 8 (LE41; ref. 32) or an anti-TAG mAb, PAb412 (31).

RESULTS

Generation of *H-2K^b-tsA58* Transgenic Mice. A hybrid construct containing the *H-2K^b* 5' promoter sequences fused to the tsA58 early region gene, which encodes both TAG and the small tumor antigen (Fig. 1 Upper), was microinjected into fertilized oocytes from (CBA/Ca \times C57BL/10) F₁ mice. After reimplantation, 88 mice were born, of which 34 carried one to five copies of the gene. RNA from a variety of tissues from one nontransgenic and three transgenic animals was analyzed by Northern blot analysis with an SV40 early region-specific probe (Fig. 1 Lower). RNA extracted from tissues of transgenic mice contained various amounts of a 2.5-kb RNA species, while no tsA58 TAG RNA was detected in tissues of the nontransgenic mouse; thymus and liver showed the highest level of expression, while brain showed the lowest.

Fibroblasts Derived from *H-2K^b-tsA58* Transgenic Mice Are Conditionally Immortal. Skin fibroblasts from normal and founder transgenic animals 2–10 weeks old were placed in culture at 33°C, the permissive temperature for the tsA58 TAG, in the presence of IFN- γ (to increase expression from the *H-2K^b* promoter; refs. 21–23). Fibroblasts derived from nontransgenic mice stopped dividing *in vitro* within a small number of passages. This cessation of division, which has been termed both "senescence" and "crisis," occurs reproducibly in fibroblasts that do not express immortalizing genes. In contrast, fibroblasts derived from most transgenic mice continued to grow for as long as the cultures were maintained under appropriate conditions (see below).

Detailed analysis of skin fibroblast cultures for conditionality of growth revealed three families of cultures, depending upon the ability of cells to grow in fully permissive, semipermissive, and nonpermissive conditions. Permissive conditions were defined as growth at 33°C in the presence of IFN- γ ; semipermissive conditions, growth at 33°C in the absence of IFN- γ or 39.5°C in the presence of IFN- γ ; and

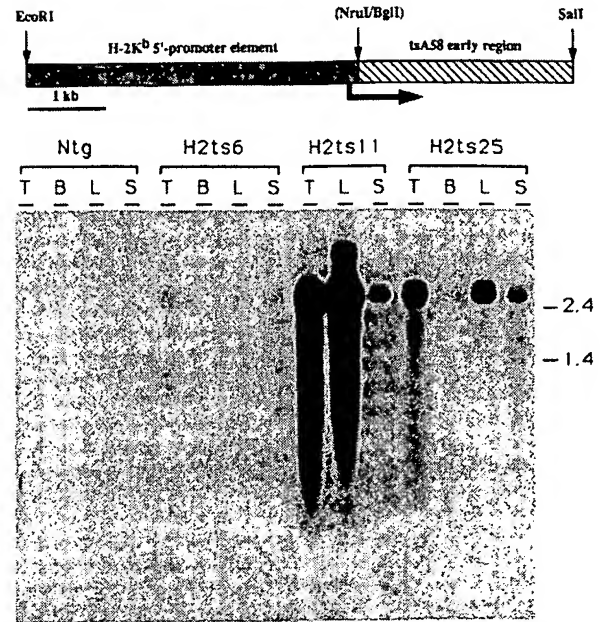


FIG. 1. (Upper) Schematic representation of the *H-2K^b-tsA58* fragment. Size in kb is indicated. (Lower) Northern blot analysis shows TAG mRNA at various levels in thymus (lanes T), brain (lanes B), liver (lanes L), and skin (lanes S) of different *H-2K^b-tsA58* transgenic mice. Loading of RNA was checked by hybridization of the same filter with an actin probe (not shown). Size in kb is indicated on the right. Ntg, nontransgenic.

nonpermissive conditions, growth at 39.5°C in the absence of IFN- γ (Fig. 2).

In the first family of cultures, growth was fully conditional and only occurred under permissive conditions. If cells were grown at 39.5°C and/or were grown in the absence of IFN- γ , cell division did not occur either in standard growth assays or in colony-forming assays (Fig. 2). These fibroblasts thus behaved as expected from previous studies in which rat embryo fibroblasts were conditionally immortalized with tsA58 TAG by retroviral infection (15). All cultures derived from different individuals within this strain yielded identical results.

In a second family of cultures, optimal growth was obtained under fully permissive conditions, a lesser degree of growth was seen under semipermissive conditions, and no growth occurred under nonpermissive conditions. In the third family, cell growth did not completely cease even under nonpermissive conditions, although the best growth was seen under fully permissive conditions.

The conditionality of growth seen in fibroblasts derived from transgenic animals was correlated with the levels of tsA58 TAG (Fig. 2e). In all cultures, the level of tsA58 TAG was reduced by temperature increase and/or by removal of IFN- γ . Interestingly, when the most conditional cultures (those derived from progeny of mouse H2ts6) were grown at 33°C in the absence of IFN- γ , a condition where these cells did not grow, low levels of TAG were still detected (Fig. 2e).

Thymic Hyperplasia in *H-2K^b-tsA58* Transgenic Mice. Enlarged thymuses occurred in all transgenic animals, a tissue-specific hyperplasia that previously has been observed in transgenic mice harboring wild-type TAG (33, 34); the time of onset of hyperplasia (2–20 weeks) was correlated with the levels of TAG mRNA (see Fig. 1 Lower). Despite the thymic enlargement, there was no evidence for malignant transformation of this tissue as judged by the following criteria: both lobes of the thymus were equally enlarged in all animals examined, and histological and immunohistochemical examination revealed

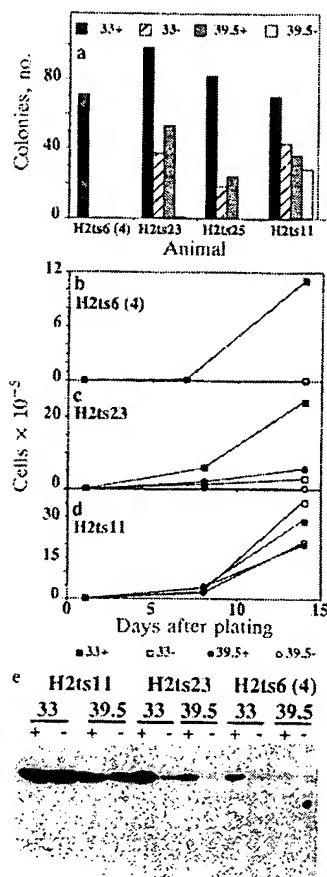


FIG. 2. (a-d) Cloning and proliferation analysis of skin fibroblasts from *H-2K^b-tsA58* transgenic mice reveals three families of cells. In family 1 (mouse H2ts6-4), cloning and proliferation are fully conditional and only occur when cells are grown at 33°C in the presence of IFN-γ (33+) (a and b). In family 2 (mice H2ts23 and H2ts25), optimal results were obtained when cells were grown at 33°C in the presence of IFN-γ (33+), no growth occurred at 39.5°C in the absence of IFN-γ (39.5-), and intermediate levels of growth were seen in the semipermissive conditions of 33°C, IFN-γ⁻ (33-) or 39.5°C, IFN-γ⁺ (39.5+) (a and c). In family 3 (mouse H2ts11), growth occurred in all conditions but was most vigorous at 33°C in the presence of IFN-γ (a and d). A reduced cloning efficiency and rate of cell growth was seen in semipermissive conditions, and a still greater reduction was seen in fully nonpermissive conditions. (e) Western blot analysis of skin fibroblasts shows that the levels of TAG are correlated with the conditionality of *in vitro* growth. The most conditional cells (derived from progeny of H2ts6) contained the lowest levels of TAG, and the least conditional cells (derived from H2ts11) showed the highest levels of TAG. In all cases, the level of TAG present increased upon addition of IFN-γ to the cultures and decreased upon shift to 39.5°C.

extensive growth of epithelial cells and the presence of apparently normal thymocyte populations, as determined by fluorometric cytometry (not shown). In addition, demarcation between cortical and medullary regions was still maintained even after prolonged hyperplastic growth (Fig. 3). Moreover, dissociated cells obtained from enlarged thymuses did not yield tumors in syngeneic recipients even when 10⁷ cells were injected s.c. or i.p., and recipient animals were sacrificed after 3 months (unpublished observations). Finally, analysis of T-cell receptor β chain gene rearrangements by Southern blot of DNA from enlarged thymuses suggested polyclonal expansion of thymocyte populations (Y.T., unpublished observations), in contrast to the oligoclonal expansion observed in mice that harbor a hybrid *Thy-1-myc* gene (35). As it is possible that the

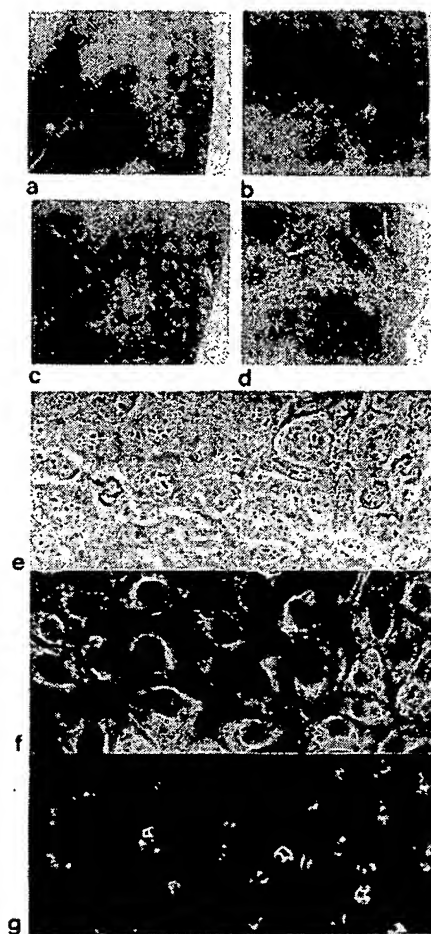


FIG. 3. (a-d) Histological analysis of thymic tissues from a nontransgenic mouse (a) and from H2ts6 mice 2 months (b), 4 months (c), and 6 months (d) old. The thymuses of H2ts6 mice up to 2 months old appeared to be identical to those of normal mice (a and b), exhibiting normal ratios between cortex (darkly stained tissue) and medulla (lightly stained tissue). The thymic architecture in 4-month-old mice (c) showed signs of disruption, while areas with cortical or medullary characteristics were still maintained. In thymic tissue of 6-month-old H2ts6 mice, extensive lightly staining areas were evident even in subcapsular regions. However, even in these organs a clear demarcation between "cortical" and "medullary" areas was still maintained. (e-g) Immunofluorescent staining of thymic epithelial cells. Cells from an adherent cell line (7P) derived from the thymus of H2ts23 were photographed under: phase optics, showing flattened cells with tightly apposed borders (e); optics with fluorescein isothiocyanate, showing filamentous cytoplasmic staining characteristic of keratins (f); and optics with rhodamine isothiocyanate, indicating the presence and nuclear localization of TAG in virtually all cells (g). (×200.)

large number of highly proliferative and hyperplastic cells in the thymus represents a target in which secondary cooperating mutations might occur, we cannot exclude the possibility that a very small number of cells within the hyperplastic thymus have undergone transformation.

In heterozygous progeny of one mouse (H2ts6), the thymus displayed normal development for extended periods, with the first histological appearance of hyperplasia seen at 4 months (Fig. 3). Homozygote offspring of H2ts6 developed thymic hyperplasia earlier (unpublished observations), in agreement with the view that the time of onset of this abnormality is correlated with TAG levels. Thymic hyperplasia was occasionally seen in conjunction with enlargement of peripheral lymphoid organs (spleen, lymph nodes), but these tissues

maintained their normal histological architecture. Macroscopic evidence of liver abnormalities was seen only in one animal even though levels of transgene expression in the liver were comparable to those in the thymus (Fig. 1 *Lower*).

Conditionally Immortal Lines of Cytokeratin⁺ Thymic Epithelial Cells. Thymuses of transgenic mice readily yielded conditionally immortal cultures containing cells of both epithelial and fibroblastic morphologies, both of which could be readily cloned. Clones that exhibited epithelial-like morphologies expressed cytokeratin (Fig. 3). Both cytokeratin⁺ and cytokeratin⁻ clones showed conditional growth. Cells grew optimally in fully permissive conditions and did not grow in nonpermissive conditions (Fig. 4). Thus, we were able to readily derive conditionally immortal lines of epithelial cells and of fibroblasts from these mice.

Dose Dependence of Skin Fibroblasts Derived from H2ts6 to IFN- γ . The establishment of a colony of *H-2K^b-tsA58* transgenic mice has allowed us to begin using these animals to study more detailed aspects of TAG function. In particular, observations that fibroblasts derived from progeny of H2ts6 showed a relatively low level of TAG expression at 33°C in the presence or absence of IFN- γ (although expression was clearly higher in the presence of IFN- γ ; Fig. 2e) suggested that with this animal it might be possible to observe dramatic alterations in cell growth as a result of small changes in the level of this gene product.

Fibroblasts derived from progeny of H2ts6 mice showed promotion of cell growth by IFN- γ at levels as low as 1 unit/ml (Fig. 5). Analysis by colony formation and by cell number analysis showed that addition of IFN- γ at 100 units/ml to these cultures only increased the frequency of colony formation 3.5-fold in comparison with that seen in the presence of IFN- γ at 1 unit/ml and was only 40% increased over that achieved with IFN- γ at 10 units/ml. The difference in TAG levels at the different doses of IFN- γ was not large, with 1 unit/ml causing a 2.5-fold increase over basal levels of TAG and 100 units/ml causing an \approx 6-fold increase over basal levels of TAG.

DISCUSSION

We have generated transgenic mice that have stably integrated the SV40 mutant strain tsA58 thermolabile TAG gene,

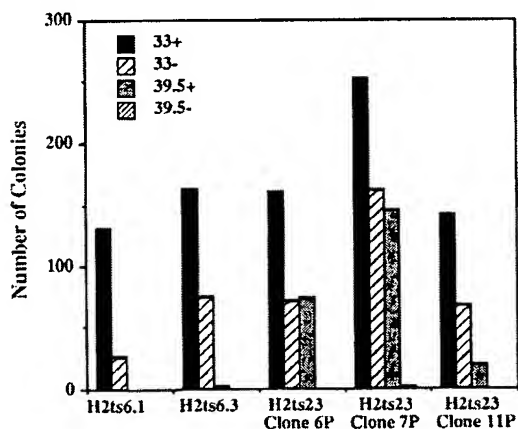


FIG. 4. Analysis of growth of thymic adherent cells by colony formation demonstrates that these cells exhibited conditional growth *in vitro*. Optimal growth occurred under the fully permissive condition of 33°C, IFN- γ ⁺ (33+) in all cases, and no colony formation occurred in nonpermissive conditions. Cultures of cells from H2ts6.1 and H2ts6.3 were derived from the thymuses of two separate progeny of founder mouse 6, and cultures of H2ts23 clones 6P, 7P, and 11P are three separate clonal cultures derived from the thymus cells of animal H2ts23; the clones 6P and 7P were morphologically epithelial, whereas clone 11P was fibroblastic. 33+, 33°C and IFN- γ ⁺; 33-, 33°C and IFN- γ ⁻; 39.5+, 39.5°C and IFN- γ ⁺; 39.5-, 39.5°C and IFN- γ ⁻.

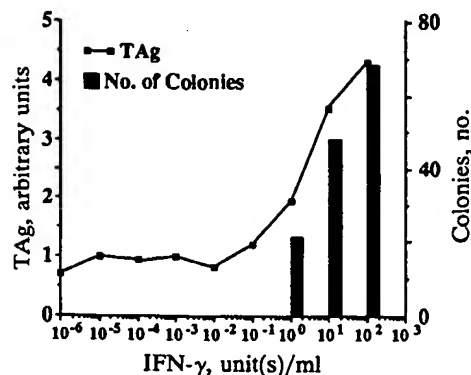


FIG. 5. Maintenance of growth of H2ts6-derived fibroblasts requires low levels of IFN- γ . No colonies were obtained in the absence of IFN- γ , but the presence of as little as 1 unit of IFN- γ per ml was sufficient to allow colony formation. Determination of the level of TAG by Western blot analysis coupled with densitometry showed that the increase in colony formation was associated with no more than a 2.5-fold increase in the level of TAG.

a conditional immortalizing gene, under the control of the inducible 5' flanking promoter of the mouse *H-2K^b* gene. The tsA58 TAG gene product is functional at the permissive temperature of 33°C but is rapidly degraded at the nonpermissive temperature of 39.5°C (13, 15). The *H-2K^b* promoter is active in a wide variety of tissues at various levels (18–21), and expression can be increased above basal levels in most cells by exposure to IFN (21–23). Fibroblasts and thymic stromal cells derived from the *H-2K^b-tsA58* transgenic mice showed conditional proliferation that could be modulated both with temperature and by application of IFN- γ ; cells from all mice grew optimally at 33°C in the presence of IFN- γ . Founder animal H2ts6, whose progeny yielded fibroblast cultures whose growth in cloning assays was completely dependent upon the permissive temperature and the presence of IFN- γ , has bred successfully to homozygosity to yield a strain of *H-2K^b-tsA58* transgenic mice.

The assay system used to examine conditionality of immortalization was based on results of previous studies in which tsA58 TAG was introduced into fibroblasts by retroviral infection (15). These conditionally immortalized fibroblasts grew indefinitely when maintained at 33°C but rapidly ceased proliferation when switched to 39.5°C. Cells derived from *H-2K^b-tsA58* transgenic mice behaved similarly. Skin cells from these mice grown *in vitro* at 33°C in the presence of IFN- γ readily yielded fibroblast cultures from all transgenic animals. Shift to semipermissive conditions of growth (i.e., 33°C/IFN- γ ⁻ or 39.5°C/IFN- γ ⁺) was sufficient to eliminate growth of cells derived from the H2ts6 strain of mice. In all other cases, shift to semipermissive conditions was associated with a reduction in cell growth but not a cessation of growth. Cultures from almost all animals ceased growth when shifted to nonpermissive conditions—i.e., 39.5°C, IFN- γ ⁻. Moreover, in the cultures (family 3 in Fig. 3) in which growth occurred after temperature increase to 39.5°C in the absence of IFN- γ , this growth was still less vigorous than that seen in semipermissive conditions. It should be noted that all cultures established from the same founder mouse, or strain of mice, exhibited identical characteristics.

Determination of the amount of TAG present in different cultures by Western blot analysis showed a direct correlation between the amount of TAG present and the growth potential of the cells. Cells in which only small amounts of TAG were produced showed stringent growth regulation, while cultures expressing high levels of TAG showed poor growth regulation. It was also clear that only small increases of TAG were needed to maintain immortalization, in that we saw only a

2.5-fold difference in levels of TAg between untreated cultures and those grown in the presence of IFN- γ at 1 unit/ml, yet only the cultures receiving the IFN- γ were able to generate colonies in a limiting dilution assay.

Conditional immortalization and the ability to readily generate rapidly growing cultures were also seen with cells derived from thymuses of transgenic mice. As with skin fibroblasts, optimal growth of the thymic cultures occurred at 33°C in the presence of IFN- γ , was reduced in semipermissive conditions, and was reduced still further in nonpermissive conditions. Interestingly, thymic cells derived from H2ts6 animals did not grow at 39.5°C in the presence of IFN- γ but did grow at 33°C in the absence of IFN- γ . This pattern of growth may reflect a higher constitutive level of transcription from the *H-2K^b* promoter in the thymic cells and/or a greater sensitivity of thymic cells to the action of TAg as compared with fibroblasts. The probable relevance of the first explanation is supported by observations that *in vivo* expression of the transgene in the thymus was generally higher than in other organs, while the relevance of the second explanation is supported by observations that the liver—the one organ in which transgene expression was similar to that of the thymus—rarely showed abnormal growth. The different effects of the transgene on thymus and liver *in vivo* suggest that cell types can differ in their susceptibility to the action of TAg.

Long-term survival of the transgenic mice was correlated with the level of conditionality of growth of the *in vitro* cultures. The only visible cause of physical distress found repeatedly was thymic enlargement. This enlargement seemed to represent hyperplastic growth rather than malignancy because thymic histology, T-cell repertoire, and T-cell clonality were all normal, and cells derived from enlarged thymuses did not generate tumors in syngeneic recipients. Although all populations of the thymus were expanded *in vivo*, only adherent cell cultures were readily obtained in long-term culture, in contrast to cells derived from *Thy-1-myc* mice (35). The generalized hyperplasia of thymic populations we have observed is similar to that seen in transgenic mice when wild-type TAg gene expression was regulated by its own early region promoter (35) or by the promoter from growth hormone-releasing factor gene (34). It differs from the hyperplasia observed in mice where the *H-2K^b* promoter was used to drive expression of the *fos* oncogene in which expansion of the epithelial component, but not of the lymphoid component, was seen (36).

Although all animals eventually succumbed to thymic hyperplasia, thus indicating that the transgene was not fully inactivated *in vivo*, the H2ts6 heterozygotes survived to the age of 6 months and homozygotes survived to the age of 3 months. Both heterozygotes and homozygotes breed normally in brother/sister matings.

The presence of a viable strain of transgenic mice harboring the *H-2K^b-tsA58* transgene will allow us to determine whether this approach to cell line production is applicable to tissues—including embryonic tissues—other than skin and thymus. As SV40 TAg can immortalize a wide range of cell types (4, 5, 7, 9, 11, 15–17) and IFN induces the expression of class I genes in a variety of tissues (21–23), the *H-2K^b-tsA58* transgenic mice may allow direct derivation of cell lines from a wide variety of different tissues and cell types. Moreover, the ability to remove the immortalizing function of the tsA58 TAg in cells derived from these transgenic mice by temperature shift up may allow us to generate cell lines that are not only conditional in their growth but also may be capable of differentiating into different types of end-stage cells (see, e.g., refs. 4 and 16). Finally, as cells prepared from these transgenic mice are genetically homogeneous, can be

prepared in large numbers, and can be synchronously exposed to interferon *in vitro*, these cells will allow study of the acute effects of SV40 TAg expression on division and differentiation in the absence of extensive *in vitro* growth and application of drug selection.

We thank A. Mellor and H. Land for plasmids, E. B. Lane for antibody LE41, Z. Ikram and A. Powell for technical assistance, and B. Wagner for stimulating discussions. P.A. is supported by a grant from Action Research for the Crippled Child; Y.T., N.Y., and D.K. are supported by the Medical Research Council; and P.S.J. and M.D.N. are supported by the Ludwig Institute for Cancer Research.

1. Darnell, J., Lodish, H. & Baltimore, D. (1986) *Molecular Cell Biology* (Scientific American Books, New York).
2. Glover, D. (1987) *DNA Cloning: A Practical Approach* (IRL, Oxford, U.K.).
3. Jat, P. S. & Sharp, P. A. (1986) *J. Virol.* 59, 746–750.
4. Frederiksen, K., Jat, P. S., Valtz, N., Levy, D. & McKay, R. (1988) *Neuron* 1, 439–448.
5. Bayley, S. A., Stones, A. J. & Smith, C. G. (1988) *Exp. Cell Res.* 177, 232–236.
6. Birren, S. J. & Anderson, D. J. (1990) *Neuron* 4, 189–201.
7. Burns, J. S., Lemoine, L., Lemoine, N. R., Williams, E. D. & Wynford-Thomas, D. (1989) *Br. J. Cancer* 59, 755–760.
8. Evrard, C., Galiana, E. & Rouget, J. (1988) *J. Neurosci. Res.* 21, 80–87.
9. Lemoine, N. R., Mayale, E. S., Jones, T., Sheer, D., McDermid, S., Kendall Taylor, P. & Wynford-Thomas, D. (1989) *Br. J. Cancer* 60, 897–903.
10. Santerre, R. F., Cook, R. A., Crisel, R. M. D., Sharp, J. D., Schmidt, R. J., Williams, D. C. & Wilson, C. P. (1981) *Proc. Natl. Acad. Sci. USA* 78, 4339–4343.
11. Williams, D. A., Rosenblatt, M. F., Beier, D. R. & Cone, R. D. (1988) *Mol. Cell. Biol.* 8, 3864–3871.
12. Ridley, A., Patterson, H., Noble, M. & Land, H. (1988) *EMBO J.* 7, 1635–1645.
13. Tegtmeyer, P. (1975) *J. Virol.* 15, 613–618.
14. Petit, C. A., Gardes, M. & Feunteun, J. (1989) *Virology* 127, 74–82.
15. Jat, P. S. & Sharp, P. A. (1989) *Mol. Cell. Biol.* 9, 1672–1681.
16. Zaret, K. S., Dipersio, C. M., Jackson, D. A., Montigny, W. J. & Weinstat, D. L. (1988) *Proc. Natl. Acad. Sci. USA* 85, 9076–9080.
17. Randa, L. R., Caton, Y., Jha, K. K., Kaplan, P., Li, G., Tragano, F. & Ozer, H. L. (1989) *Mol. Cell. Biol.* 9, 3093–3096.
18. Weiss, E. H., Mellor, A., Golden, L., Fahrner, K., Simpson, E., Hurst, J. & Flavell, R. A. (1983) *Nature (London)* 301, 671–674.
19. Baldwin, A. S., Jr., & Sharp, P. A. (1987) *Mol. Cell. Biol.* 7, 305–313.
20. Kimura, A., Israël, A., Le Bail, O. & Kourilsky, P. (1986) *Cell* 44, 261–272.
21. David-Wattine, B., Israël, A. & Kourilsky, P. (1990) *Immunol. Today* 11, 286–292.
22. Wallach, D., Fellous, M. & Revel, M. (1982) *Nature (London)* 299, 833–836.
23. Israël, A., Kimura, A., Fournier, A., Fellous, M. & Kourilsky, P. (1986) *Nature (London)* 322, 743–746.
24. Kollias, G., Spanopoulou, E., Grosveld, F., Ritter, M., Beech, J. & Morris, R. (1987) *Proc. Natl. Acad. Sci. USA* 84, 1492–1496.
25. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), 2nd Ed.
26. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
27. Noble, M., Fok-Seang, J. & Cohen, J. (1984) *J. Neurosci.* 4, 1892–1903.
28. Efrats, S., Linde, S., Kofod, H., Spector, D., Delannoy, M., Grant, S., Hanahan, D. & Backesov, S. (1988) *Proc. Natl. Acad. Sci. USA* 85, 9037–9041.
29. Mellon, P. L., Windle, J. J., Goldsmith, P. C., Padola, C. A., Roberts, J. L. & Weiner, R. I. (1990) *Neuron* 5, 1–10.
30. Hammond, J. P., Baetge, E. E., Behringer, R. R., Brinster, R. L., Palmiter, R. D. & Messing, A. (1990) *Neuron* 4, 775–782.
31. Harlow, E., Crawford, L. V., Pim, D. C. & Williamson, N. C. (1981) *J. Virol.* 39, 861–869.
32. Lane, E. B. (1982) *J. Cell Biol.* 92, 665–673.
33. Botteri, F. M., Van der Putten, H., Wong, D. F., Sauvage, C. A. & Evans, R. M. (1987) *Mol. Cell. Biol.* 7, 3178–3184.
34. Brinster, R. L., Chen, H. Y., Messing, A., Van Dyke, T., Levine, A. J. & Palmiter, R. D. (1984) *Cell* 37, 367–379.
35. Spanopoulou, E., Early, A., Elliot, J., Crispe, N., Ladyman, H., Ritter, M., Watt, S., Grosveld, F. & Kourilsky, D. (1989) *Nature (London)* 342, 185–189.
36. Rüther, U., Müller, W., Sumida, T., Tokuhisa, T., Rajewsky, K. & Wagner, E. F. (1988) *Cell* 53, 847–856.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☒ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.